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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 38/39	A1	(11) International Publication Number: WO 00/59532 (43) International Publication Date: 12 October 2000 (12.10.00)
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(54) Title: THE USE OF DOMAINS OF TYPE IV COLLAGEN T INHIBIT ANGIOGENESIS AN TUMOUR GROWTH (57) Abstract The instant invention provides methods and kits for inhibiting angiogenesis, tumor growth and metastasis, and endothelial cell interactions with the extracellular matrix, involving contacting the tumor, animal tissue, or endothelial cells with an amount effective to inhibit angiogenesis, tumor growth and metastasis, or endothelial cell interactions with the extracellular matrix of an antagonist of specific integrin receptors.		

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THE USE OF DOMAINS OF TYPE IV COLLAGEN TO INHIBIT ANGIOGENESIS AND TUMOUR GROWTH

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Cross Reference

This application claims priority to U.S. Provisional Application Serial No. 60/127,391 filed April 1, 1999, and is a continuation in part of U.S. Application Serial No. 09/277,665, filed March 26, 1999, which is a continuation in part of U.S. Application Serial No. 09/183,548 filed October 30, 1998, which is a continuation of 10 08/800,965 filed February 18, 1997, now U.S. Patent No. 5,856,184.

Field of the Invention

This invention relates to methods and kits for inhibiting angiogenesis, tumor 15 growth and metastasis, and endothelial cell interactions with the extracellular matrix.

Background of the Invention

Angiogenesis, the process of formation of new blood vessels, plays an important role in physiological processes such as embryonic and postnatal 20 development, as well as in wound repair. Formation of blood vessels can also be induced by pathological processes involving inflammation (e.g., diabetic retinopathy and arthritis) or neoplasia (e.g., cancer) (Folkman, 1985, *Perspect. Biol. Med.*, 29, 10). Neovascularization is regulated by angiogenic growth factors secreted by tumor or normal cells as well as the composition of the extracellular matrix and by the activity of 25 endothelial enzymes (Nicosia and Ottinetti, 1990, *Lab. Invest.*, 63, 115).

During the initial stages of angiogenesis, endothelial cell sprouts appear through gaps in the basement membrane of pre-existing blood vessels (Nicosia and Ottinetti,

1990, *supra*; Schoefl, 1963, Virehous Arch, Pathol. Anat. 337, 97-141; Ausprunk and Folkman, 1977, Microvasc. Res. 14, 53-65; Paku and Paweletz, 1991, Lab. Invest. 63, 334-346). As new vessels form, their basement membrane undergoes complex structural and compositional changes that are believed to affect the angiogenic response
5 (Nicosia, et. al., 1994, Exp. Biology, 164, 197-206). Early planar culture models have shown that basement membrane molecules modulate the attachment, migration, proliferation, and organizational behavior of endothelial cells (Nicosia, et. al., 1994, *supra*). More recent studies with three-dimensional aortic culture models that more closely simulate angiogenic conditions during wound healing in vivo suggest that the
10 basement membrane is a dynamic regulator of angiogenesis, and its function varies according to its molecular components (Nicosia, 1994, *supra*).

A common feature of all solid tumor growth is the requirement for a blood supply. Therefore, numerous laboratories have focused on developing anti-angiogenic compounds based on growth factors and their receptors. While this approach has led to
15 some success, the number of growth factors known to play a role an angiogenesis is large. Therefore, the possibility exists that growth factor antagonists may have only limited use in treating cancer since tumors and associated inflammatory cells likely produce a wide variety of factors that can induce angiogenesis.

In this regard, a strategy that targets a common feature of angiogenesis, such as
20 endothelial cell adhesion to the extracellular matrix (ECM), might be expected to have a profound physiological impact on tumor growth in humans. This notion is supported by the fact that RGD-containing antagonists of the $\alpha v \beta 3$ integrin ECM cell adhesion receptor can block angiogenesis. (U.S. Patent No. 5,766,591) Furthermore, the $\alpha v \beta 3$ integrin is expressed most prominently on cytokine -activated endothelial and smooth

muscle cells and has been shown to be required for angiogenesis. (Varner et al., Cell Adhesion and Communication 3:367-374 (1995); Brooks et al., Science 264:569-571 (1994)). Based on these findings, a potentially powerful new approach to anti-angiogenic therapy might be to specifically target critical regulatory domains within
5 distinct ECM components.

The basement membrane (basal lamina) is a sheet-like extracellular matrix (ECM), which is a basic component of all tissues. The basal lamina provides for the compartmentalization of tissues, and acts as a filter for substances traveling between tissue compartments. Typically the basal lamina is found closely associated with an
10 epithelium or endothelium in all tissues of an animal including blood vessels and capillaries. The basal lamina components are secreted by cells and then self assemble to form an intricate extra-cellular network. The formation of biologically active basal lamina is important to the development and differentiation of the associated cells.

Type IV collagen has been shown to be a major structural component of
15 basement membranes. The protomeric form of type IV collagen is formed as a heterotrimer made up from a number of different subunit chains called $\alpha 1(IV)$ through $\alpha 6(IV)$. Up to now, six genetically distinct α -chains belonging to two classes with extensive homology have been identified, and their relative abundance has been demonstrated to be tissue specific. The type IV collagen heterotrimer is characterized
20 by three distinct structural domains: the non-collagenous (NC1) domain at the carboxyl terminus; the triple helical collagenous domain in the middle region; and the 7S collagenous domain at the amino terminus. (Martin, et. al., 1988, Adv. Protein Chem. 39:1-50; Gunwar, et. al. 1991, J. Biol. Chem. 266:14088-14094).

The ability to express recombinant α (IV) NC1 domains provides the opportunity to study the effect of specific domains on many biological processes, such as angiogenesis, tumor metastasis, cell binding to basement membranes, and assembly of Type IV collagen molecules.

5

Summary of the Invention

The instant invention provides methods and kits for inhibiting angiogenesis, tumor growth and metastasis, and endothelial cell interaction with the extracellular matrix, each method comprising contacting the tumor, animal tissue, or endothelial cells with antagonists of specific integrin receptors.

10

Brief Description of the Drawings

Figure 1 illustrates the effects of NC1 (Hexamer) and 7S domains of Type IV collagen at a 50 μ g/ml concentration on angiogenesis from mouse thoracic aorta organ cultures.

15 Figure 2 illustrates the effects of 7S domain of Type IV collagen on angiogenesis from mouse thoracic aorta organ cultures. The domain concentrations employed in this experiment were 0 μ g/ml (control); 0.5 μ g/ml; 5 μ g/ml and 50 μ g/ml.

Figure 3 illustrates the effects of NC1 (Hexamer) domain of Type IV collagen on angiogenesis from mouse thoracic aorta organ cultures. The domain concentrations employed in this experiment were 0 μ g/ml (control); 5 μ g/ml and 5 μ g/ml and 50 μ g/ml.

20

Figure 4 are photographs of mouse thoracic aorta segments embedded in Matrigel (EHS basement membrane matrix, Collaborative Biomedical Products, Bedford, MA) at 5 days of culture. Control specimen (0 μ g/ml of NC1 (Hexamer) and 7S domains)

exhibited growth of microvessels from the cultured tissue into the matrix (Figure 4A). In contrast, angiogenesis was inhibited in specimens cultured with 50 $\mu\text{g/ml}$ of 7S domain (Figure 4B) and NC1 (Hexamer) domain (Figure 4C).

Figure 5 is a graphical representation of data demonstrating the in vivo effect of IV injection of recombinant ($\alpha 1$) type IV collagen monomer on angiogenesis using fibrin implants in rats.

Figure 6 is a graphical representation of data demonstrating that the recombinant ($\alpha 1$) and ($\alpha 2$) NC1 monomers inhibit the bFGF-induced increase in angiogenic index in vivo.

Figure 7 is a graphical representation of demonstrating the dose response effect of recombinant ($\alpha 2$) NC1 monomer on the bFGF-induced increase in total blood vessel branch points in vivo.

Figure 8 is a graphical representation of data demonstrating the dose response effect of recombinant ($\alpha 2$) NC1 monomer on the bFGF-induced increase in angiogenic index in vivo.

Figure 9 is a graphical representation of data demonstrating the dose response effect of recombinant ($\alpha 2$) NC1 monomer on the bFGF-induced increase in angiogenic index in vivo.

Figure 10 is a graphical representation of data demonstrating the effect of recombinant ($\alpha 1$) and ($\alpha 2$) NC1 monomers on mean CS-1 melanoma tumor weight in vivo.

Figure 11 is a graphical representation of data demonstrating the dose response effect of recombinant ($\alpha 2$) NC1 monomer on mean CS-1 melanoma tumor weight in vivo.

Figure 12 is a graphical representation of data demonstrating the effect of recombinant ($\alpha 1$), ($\alpha 2$), and ($\alpha 4$) NC1 monomers on mean HT1080 tumor weight in vivo.

Figure 13 is a graphical representation of data demonstrating the effect of recombinant ($\alpha 1$), ($\alpha 2$), ($\alpha 3$) and ($\alpha 5$) NC1 monomers on mean HEP-3 tumor weight in vivo.

Figure 14 is a graphical representation of data demonstrating human endothelial cell adhesion to immobilized NC1 α monomers.

- 5 Figure 15 is a graphical representation of data demonstrating the effect of soluble $\alpha 1$ and $\alpha 2$ NC1 monomers on human endothelial cell adhesion to pepsinized collagen type IV.

Figure 16 is a graphical representation of data demonstrating the effect of isolated recombinant NC1 monomers on human endothelial cell migration in vitro.

- 10 Figure 17 A-F provides the sequences of each type IV collagen α chain monomer.

Figure 18 is a graphical representation of data demonstrating the effect of monoclonal antibodies against various integrins on human endothelial cell adhesion to recombinant the ($\alpha 2$) NC1 domain.

- Figure 19 is a graphical representation of data demonstrating human endothelial cell
15 adhesion to the recombinant ($\alpha 1$) NC1 domain.

Description of the Preferred Embodiments

- Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press),
20 *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutscher, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press,

San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique*, 2nd Ed. (R.I. Freshney. 1987. Liss, Inc. New York, NY), and *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

As used herein, the term Type IV collagen domain encompasses the group of
5 molecules including the non-collagenous NC1 domain (Hexamer) and 7S collagenous domains, as well as NC1 α chain monomers.

The invention comprises methods for using Type IV collagen NC1 α -monomers (ie: $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 6$), which are defined to include such monomers isolated from any multicellular organism or produced via recombinant protein expression from a gene
10 encoding such a monomer from any multicellular organism, and also to encompass various modifications, additions, and/or deletions to such monomers.

In one aspect, the present invention provides methods and kits for inhibiting angiogenesis in an animal tissue comprising contacting the tumor or animal tissue with an amount effective to inhibit angiogenesis of a polypeptide composition comprising one
15 or more isolated type IV collagen NC1 α chain monomers selected from the group consisting of $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 6$ NC1 chain monomers.

In another aspect, the present invention provides methods and kits for inhibiting tumor growth in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor growth of a polypeptide composition comprising one or more
20 isolated type IV collagen NC1 α chain monomers selected from the group consisting of $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 6$ NC1 chain monomers.

In another aspect, the present invention provides methods and kits for inhibiting tumor metastasis in tissue comprising contacting the tumor or tissue with an amount effective to inhibit metastasis of a polypeptide composition comprising one or more

isolated type IV collagen NC1 α chain monomers selected from the group consisting of $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 6$ NC1 chain monomers.

In a further aspect, the present invention provides methods and kits for inhibiting endothelial cell interactions with the extracellular matrix in tissue comprising
5 contacting the tumor or tissue with an amount effective to inhibit endothelial cell interactions with the extracellular matrix of a polypeptide composition comprising one or more isolated type IV collagen NC1 α chain monomers selected from the group consisting of $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 6$ NC1 chain monomers.

The NC1-encoding domain of each of the six α chain cDNAs has been cloned
10 into a vector for recombinant protein expression as previously described (Sado et al., Kidney Intl. 53:664-671 (1998), incorporated by reference herein in its entirety). The vectors are used to stably transfect human kidney 293 cells, which produce the recombinant protein. The DNA and deduced amino acid sequences of the recombinant type IV collagen alpha chain monomers produced as described are shown in Figure
15 17A-F. The first 17 amino acids correspond to a BM40 signal sequence (which is cleaved from the mature protein), to facilitate protein secretion. All the secreted proteins (ie: mature proteins) start with the sequence APLA followed by the affinity tag, DYKDDDDK at the amino terminus. This tag facilitates purification and identification of the material, and does not interfere with biological activity of the
20 recombinant NC1 α chain monomers.

The type IV collagen NC1 α chain monomers can be produced by any method known in the art, including using recombinant DNA technology or biochemical peptide synthesis technology, or by isolating the NC1 domains from animal sources, such as from basement membrane sources such as bovine lens capsule and bovine kidney glomeruli.

(Peczon et al., Exp. Eye Res. 30:155-165 (1980); Langeveld et al., J. Biol. Chem. 263:10481-10488 (1988); Gunwar et al., J. Biol. Chem. 266:14088-14094 (1991))

In practicing the invention, the amount or dosage range of type IV collagen NC1 α chain monomers employed is one that effectively inhibits angiogenesis, tumor growth, tumor metastasis, and/or endothelial cell-extracellular matrix interactions. An inhibiting amount of NC1 α chain monomers that can be employed ranges generally between about 0.01 $\mu\text{g/kg}$ body weight and about 10 mg/kg body weight, preferably ranging between about 0.05 $\mu\text{g/kg}$ and about 5 mg/kg body weight.

The NC1 α chain monomers may be administered by any suitable route, including orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intraarterial, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally. In preferred embodiments, the NC1 α chain monomers are administered intravenously or subcutaneously.

The NC1 α chain monomers may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The NC1 α chain monomers of the invention may be applied in a variety of solutions. Suitable solutions for use in accordance with the invention are sterile, dissolve sufficient amounts of the NC1 α chain monomers, and are not harmful for the proposed application.

The NC1 α chain monomers may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

For administration, the NC1 α chain monomers are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanolic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Example 1 – In Vitro Effect on Angiogenesis

With modifications, the procedures of Nicosia and Ottinetti (1990), *supra*, and Nicosia, et. al. (1994), *supra*, were utilized for experiments designed to test the effect of Type IV collagen on angiogenesis under *in vitro* conditions. The model has been used to study the effects of growth factors and extracellular matrix molecules on the

angiogenic response and employs aortic rings cultures in three-dimensional collagen gels under serum-free conditions. These experiments are outlined below.

A. Methods

Experiments were performed with 1-3 month old Swiss Webster male mice.

- 5 Following anesthesia, the thoracic aorta was excised under aseptic conditions and transferred to sterile MCDB 131 sterile growth medium (Clonetics, San Diego, CA) containing antibiotics. Fat was dissected away from the aorta and approximately six to eight 1 mm thoracic segments were obtained from each specimen. Segments were transferred to 48 well tissue culture plates. The wells of these plates were layered with
- 10 100 microliters of Matrigel (EHS basement membrane, Collaborative Biomedical Products, Bedford, MA) prior to transfer of the aortic segments. The Matrigel was diluted 1:1 with MCDB 131 growth medium prior to use. The segments were centered in the wells and an additional 100 microliters of Matrigel was then placed over the specimens. The aortic segments were therefore embedded in the basement membrane
- 15 matrix. Each well then received 300 microliters of MCDB 131 growth medium. The plates were placed in an incubator maintained at 37° C with 5% CO₂. Specimens were observed daily over a 7 day period. Newly growing microvessels were counted using an inverted phase microscope at various times during the culture period, but data is expressed at 3 and 5 days of culture. To test for the effect of Type IV collagen on
- 20 angiogenesis, domains at known concentrations were mixed with the Matrigel and with the MCDB 131 growth medium. Fresh MCDB 131 growth medium (plus and minus collagen domains) was changed every 3 days.

B. Results

After establishing the time course of angiogenesis under control conditions (Matrigel plus MCDB 131 growth medium), experiments were performed using various concentrations of Type IV collagen (isolated from bovine lens) NC1 (hexamer) and 7S domains. Data represents the analysis of at least 3 specimens per experimental condition. In the first experiment (**Figure 1**), analysis indicated that at a concentration of 50 µg/ml, NC1 domain and 7S domain significantly inhibited angiogenesis as monitored at 3 and 5 days of culture. In the second experiment, various concentrations of these domains were analyzed. As indicated in **Figure 3**, 7S domain at 50 µg/ml again significantly inhibited angiogenesis at 3 and 5 days. Inhibition was reduced at 5 and 0.5 µg/ml concentrations. As indicated in **Figure 2**, NC1 domain was less effective in blocking angiogenesis as compared to that observed in the first experiment (**Figure 1**), although it was still effective. In addition, as compared to the 7S domain, there was less of a correlation between concentration and inhibitory action.

Figure 4A-C are photographs of mouse thoracic aorta segments embedded in Matrigel (EHS basement membrane matrix, Collaborative Biomedical Products, Bedford, MA) at 5 days of culture in the presence or absence of 50 µg/ml of Type IV collagen domains. The control specimen (no domains) exhibited growth of microvessels from the cultured tissue into the matrix (**Figure 4A**). In contrast, angiogenesis inhibition was observed in tissues cultured in the presence of 50 µg/ml of 7S (**Figure 4B**) and NC1 (Hexamer) domain (**Figure 4C**).

Example 2. Subcutaneous fibrin implant angiogenesis

Recombinant human type IV collagen NC1 ($\alpha 3$) monomer (Sado et al., Kidney International 53:664-671 (1998)) was injected intravenously in Fisher 344 rats containing fibrin implants surgically placed subcutaneously, a modified version of the method described by Dvorak et al (Lab. Invest. 57(6):673-686 (1987)). The implants
5 were then removed and directly analyzed using an inverted microscope. The analysis involved counting the number of blood vessels that had grown into the fibrin in the control and experimental group.

Briefly, 4 fibrin implants were surgically implanted subcutaneously into Fisher 344 rats (2 dorsal and 2 ventral sides). The average rat weight was approximately 125
10 grams.

Three rats (EXP) were given tail vein injections of either control (fibrin alone), 100 μ l of 100 μ g/ml of 7S domain of type IV collagen (approximately 0.80 mg/kg body weight), 100 μ l of 100 μ g/ml of type IV collagen hexamer (approximately 0.80 mg/kg body weight), or recombinant collagen type IV NC1 ($\alpha 3$) monomer at a concentration
15 of 1.26 mg/ml in PBS (120 μ g protein, or approximately 0.96 mg/kg body weight) and 3 rats (C) were given 100 μ l tail vein injections of PBS. Injections of recombinant protein were given every other day for five doses. The injection schedule was as follows:

20 Day 1: (implant day) injection and remove blood sample (EXP and C)
Day 3: Injection (EXP and C)
Day 5: Injection and remove blood sample (EXP and C)
Day 7: Injection (EXP and C)
Day 9: Injection and remove blood sample (EXP and C)
25 Day 11: Remove and fix implants (save blood sample) (EXP and C)

The results of one experiment were as follows:

2 week in vivo experiment:

Control (fibrin alone)

about 66 BV

7S domain of type IV lens collagen (100 µg/ml)	None
Hexamer of type IV lens collagen (100 µg/ml)	None
Monomer (α 3)	None

5 The results are shown as the mean number of blood vessels per implant. The results of this study demonstrate that isolated domains of type IV collagen, including the α 3 monomer, can significantly inhibit capillary growth in the in vivo fibrin clot implant model. In subsequent experiments, the inhibitory effect was occasionally seen to attenuate with time, suggesting that higher dosages or more frequent injections might
10 be even more effective.

A similar experiment was conducted using recombinant human type IV collagen NC1 (α 1) monomer (100 µl of a 1 µg/µl solution; approximately 0.80 mg/kg body weight) and comparing the number of blood vessels that had grown into the fibrin at day 11 of treatment relative to the control group. Three rats per group were analyzed
15 with each rat having 4 implants. These experiments demonstrated that administration of the α 1 monomer significantly inhibited capillary growth in the in vivo fibrin clot implant model (Figure 5).

Example 3. Recombinant NC1 (α 2) domain inhibits angiogenesis in vivo

20 We next tested the effects of systemic administration of soluble NC1 α -chain monomers in the chick embryo CAM angiogenesis assay.

Angiogenesis was induced in the CAMs of 10 day old chick embryos with bFGF as described (Brooks et al., Cell 92:391-400 (1998)). Twenty four hours later the embryos were systemically treated with various concentrations of recombinant NC1 α -
25 chain monomers, in a total volume of 100 µl of sterile phosphate buffered saline (PBS). Two days later the embryos were sacrificed and the filter discs and CAM tissues

removed. Angiogenesis was quantitated by counting the number of angiogenic blood vessel branch points in the confined area of the filter disc. The Angiogenic Index is defined as the number of branch points from experimental treatment minus control treatment.

5 In initial experiments, recombinant $\alpha 1$ or $\alpha 2$ NC1 domains were injected at a concentration of 50 μ g per embryo. At this concentration, the NC1 domains were shown to be highly toxic as demonstrated by greater than 90% embryo cell death. However, at lower doses they were well tolerated and showed potent anti-angiogenic activity. A total of 6 individual angiogenesis experiments were conducted with the
10 NC1 domains. However, in two experiments, the bFGF induction was low, making it difficult to interpret the results. The NC1 $\alpha 2$ domain appeared to be more consistent and potent than the $\alpha 1$ NC1 domain at inhibiting angiogenesis. In fact, systemic administration of 30 μ g of NC1 $\alpha 2$ consistently inhibited angiogenesis by greater than 90% (Figures 6-9), as measured by inhibition of the bFGF-induced increase in the
15 angiogenic index and the mean number of blood vessel branch points. In contrast, NC1 $\alpha 1$ domain showed variable inhibitory activity (0%-50%) throughout the experiments.

Example 4. Recombinant NC1 domain inhibits melanoma tumor growth in vivo:

20

Since the growth of all solid tumors depends on angiogenesis to provide nutrients for its continued expansion, reagents that have the capacity to inhibit angiogenesis may significantly inhibit tumor growth. Therefore, we tested the effects of recombinant NC1 domains of type IV collagen for their effects on tumor growth in

25 vivo.

To test the effects of NC1 domains on tumor growth in vivo, we utilized the chick embryo tumor growth assay. Briefly, single cell suspensions of 3 distinct tumor types were applied to the CAM of 10 day old chick embryos. The tumors included CS-1 Melanoma cells (5×10^6), HT1080 human fibrosarcoma cells (4×10^5) and Hep-3 human epidermoid carcinoma cells (2×10^5). The embryos were injected systemically with varying concentrations of NC1 α -chain monomers 24 hours later. The embryos were next allowed to incubate for a total of 7 days, at which time they were sacrificed. The resulting tumors were resected and wet weights determined. A total of 6 tumor growth assays were conducted with the 3 distinct tumor types. A single injection of 10 μ g NC1 α 2 domain inhibited CS1 melanoma tumor growth by approximately 70% relative to control (Figure 10). In similar experiments, dose response curves were completed with CS-1 tumors. Systemic administration of NC1 α 2 resulted in a dose-dependent inhibition of CS-1 melanoma tumor growth in vivo with a maximum inhibition following a single dose at 30 μ g (Figure 11). Systemic administration of NC1 α 1 also inhibited CS-1 tumor growth but it was variable and in some experiments failed to inhibit tumor growth (See Figure 10). In similar experiments, NC1 α 2 inhibited HT1080 human fibrosarcoma tumor growth by approximately 50% after a single systemic injection of 30 μ g, while NC1 α 1 and α 4 had no effect (Figure 12). Finally, systemic administration of NC1 α 2 (30.0 μ g) and α 3 inhibited Hep-3 human epidermoid carcinoma tumor growth by approximately 40% and 60% respectively, and α 1 inhibited Hep-3 tumor growth by approximately 30%, while NC1 α 5 domain failed to inhibit tumor growth (Figure 13).

We conclude from these in vivo studies that tumor growth can be inhibited by isolated NC1 α -chain monomers. These molecules can thus be used alone, or to

complement the use of existing anti-tumor agents, in providing enhanced and more effective anti-tumor therapy.

Example 5. Immobilized NC1 domains support human endothelial cell adhesion

5 In order for new blood vessels to form, endothelial cells must have the capacity to adhere and migrate through the ECM. Moreover, this endothelial cell-ECM interaction may facilitate signal transduction events required for new blood vessel formation. Therefore, since type IV-collagen is an ECM protein which is known to support cell adhesion, we tested the ability of the NC1 domains to support endothelial
10 cell attachment.

Microtiter plates were coated with 25 μ g/ml of purified NC1 domains followed by incubation with 1% bovine serum albumin (BSA) to block non-specific interactions. Human endothelial cells (ECV304) were then allowed to attach to the immobilized NC1 domains for 1 hour. Non-adherent cells were removed by washing and attached
15 cells were quantified by measuring the optical density (O.D.) of crystal violet eluted from attached cells. Data bars represent the mean \pm standard error of the O.D. from triplicate wells.

Immobilized NC1 α 2, α 3, and α 6 domains supported endothelial cell adhesion while NC1 α 1, α 4, and α 5 domains promoted little if any cell adhesion (Figure 14).
20 Soluble NC1 α 1 (α 1) and α 2 (α 2) inhibited endothelial cell adhesion to pepsinized collagen type IV by approximately 50% (Figure 15).

Taken together, these findings demonstrate that isolated, recombinant NC1 domains from the α 1, α 2, α 3, and α 6 chains of collagen type IV can mediate human endothelial cell adhesion and/or inhibit endothelial cell adhesion to ECM proteins in

vitro, and suggest that the potent anti-angiogenic and anti-tumor activity of the isolated NC1 domains is due to disruption of endothelial cell interaction with the extracellular matrix that are necessary for angiogenesis.

5 **Example 6. Endothelial Cell Migration**

Invasive cellular processes such as angiogenesis and tumor metastasis also require cellular motility. Thus we evaluated the ability of isolated NC1 domains to support human endothelial cell migration in vitro. These experiments were conducted essentially according to the methods in Brooks et al., J. Clin. Invest. 99:1390-1398
10 (1997).

The results of these experiments indicate that NC1 $\alpha 2$, $\alpha 3$, and $\alpha 6$ domains can support human endothelial cell migration in vitro, while $\alpha 1$, $\alpha 4$, and $\alpha 5$ domains showed little if any capacity to support endothelial cell migration (**FIG 16**).

15 **Example 7. Efficacy in Lewis lung in vivo tumor**

The above studies indicated that specific domains of collagen type IV can promote cell migration in vitro. Thus, we evaluated the ability of NC1 domains to support endothelial cell migration in vivo.

The α (IV) NC1 domain hexamer, isolated by enzymatic digestion of bovine
20 lens capsule basement membrane by known protocols (Peczon et al., Exp. Eye Res. 30:155-165 (1980)) was tested in the metastatic Lewis lung mouse tumor model using a standard protocol which is considered to be a good model of both metastasis and angiogenesis of lung tumors. (See for example, Teicher et al., Anticancer Res.

18:2567-2573 (1998); Guibaud et al., *Anticancer Drugs* 8:276-282 (1997); Anderson et al., *Cancer Res.* 56:715-718 (1996)).

Each study consisted of an untreated control group and six treatment groups. There were ten animals per treatment group with 40 mice in the control. In each study, all treatment was administered intravenously once every 2 days for 7 doses starting one day after tumor inoculation. Dosages of α (IV) NC1 hexamer were either 100 $\mu\text{g}/\text{mouse}$ or 200 $\mu\text{g}/\text{mouse}$. In the Lewis lung study, the tumor cell inoculum was 1×10^6 viable cells. All animals were weighed twice a week throughout the study. Starting one day after the last treatment, 5 mice were periodically sacrificed from each control group to measure pulmonary tumor burden. The experiment was terminated at day 14 when the lungs of the control animals had sufficient tumor mass to provide meaningful evaluation. At that time, the lungs of all remaining animals were excised, weighed, and the number of tumor foci greater than 2 mm in diameter counted. The resulting data showed that both dosages of α (IV) NC1 hexamer significantly reduced the number of visible lung metastases (Mann-Whitney Rank Sum Test, $p < 0.05$), with 8 visible lung metastases in the control, vs. 5 (100 $\mu\text{g}/\text{mouse}$) and 4 (200 $\mu\text{g}/\text{mouse}$), and the 100 $\mu\text{g}/\text{mouse}$ dosage reduced the lung weights from a median of 520 mg in controls to a median of 462 mg in experimental, while the median lung weight of mice treated with 200 $\mu\text{g}/\text{mouse}$ was 620 mg.

Other in vivo studies demonstrated that tumor cell metastasis to the lung can be reduced by 50% or more using intravenous injections of the Type IV collagen domains in murine B16 melanoma, human A375SM melanoma xenografts. Furthermore, injection of the NC1 hexamer also significantly reduced the number of lung tumors in separate Lewis Lung tumor studies.

Example 8. Defining the Integrin Receptor Mediating Cellular Adhesion to the NC1 domains

To define the integrin receptors that mediated cellular adhesion to the NC1 $\alpha 1$ and $\alpha 2$ domains, adhesion assays were performed as described in Example 5 in the presence or absence of function blocking monoclonal antibodies directed to specific integrins (Figures 18 ($\alpha 2$); Fig. 19 ($\alpha 1$)). These antibodies were directed against $\alpha 5 \beta 3$ integrin (anti-avb3), the $\alpha 5 \beta 5$ integrin (anti-avb5), the $\beta 1$ integrin (b1) (all described in U.S. Patent No. 5,766,591, incorporated by reference herein in its entirety), and monoclonal antibodies directed against the $\alpha 1$ (anti-a1), $\alpha 2$ (anti-a2), and $\alpha 3$ (anti-a3) integrins (purchased from Chemicon, California). These studies indicated that human endothelial cells interact with NC1 $\alpha 2$ domain primarily through $\alpha v \beta 5$ and $\alpha v \beta 3$ integrins with variable contribution from $\beta 1$ integrins (Figure 18). In similar experiments, anti- $\beta 1$ integrin antibodies showed a lesser effect on endothelial cell adhesion to NC1 $\alpha 2$, suggesting a lesser contribution of $\beta 1$ integrins to this adhesive activity. In contrast, endothelial cell adhesion promoted by NC1 $\alpha 1$ domain was mediated by integrin $\alpha 3 \beta 1$ (Figure 19).

Previous studies have demonstrated that RGD-containing antagonists of the $\alpha v \beta 3$ receptor can block angiogenesis (U.S. Patent No. 5,766,591), but the instant invention provides the first demonstration of a non-RGD containing antagonist of the $\alpha v \beta 3$ integrin that can block angiogenesis. The present study also demonstrates that antagonists of the $\alpha v \beta 5$ integrin and the $\alpha 3 \beta 1$ integrins can block angiogenesis.

Thus, the instant invention also provides methods and kits for inhibiting angiogenesis, tumor growth and metastasis, and endothelial cell interaction with the extracellular matrix, each method comprising contacting the tumor, animal tissue, or

endothelial cells with antagonists of specific integrin receptors. Specifically, the methods comprise contacting the tumor, animal tissue, or endothelial cells with one or more of the following polypeptide compositions:

- (a) a polypeptide composition comprising one or more non-RGD containing
5 integrin $\alpha v \beta 3$ antagonists; or
- (b) a polypeptide composition comprising one or more antagonists of $\alpha v \beta 5$
integrin; or
- (c) a polypeptide composition comprising one or more antagonists of $\beta 1$
integrins; or
- 10 (d) a polypeptide composition comprising one or more antagonists of $\alpha 3 \beta 1$
integrins.

We conclude from all of the above studies that angiogenesis, tumor growth and metastasis, and endothelial cell adhesion to the ECM, can be inhibited by isolated,
15 recombinant domains of type IV collagen, or by antagonists of specific integrin receptors. The present invention is thus broadly applicable to a variety of uses which include inhibition of angiogenesis and treatment of diseases and conditions with accompanying undesired angiogenesis, such as solid and blood-borne tumors including but not limited to melanomas, carcinomas, sarcomas, rhabdomyosarcoma,
20 retinoblastoma, Ewing sarcoma, neuroblastoma, osteosarcoma, and leukemia.

The invention is further applicable to treating non-tumorigenic diseases and conditions with accompanying undesired angiogenesis, including but not limited to diabetic retinopathy, rheumatoid arthritis, retinal neovascularization, choroidal neovascularization, macular degeneration, corneal neovascularization, retinopathy of

prematurity., corneal graft rejection, neovascular glaucoma., retrolental fibroplasia, epidemic keratoconjunctivitis, Vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sogrens, acne rosacea, phlyctenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi's sarcoma, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, traum, systemic lupus, polyarteritis, Wegeners sarcoidosis, scleritis, Steven's Johnson disease, radial keratotomy, sickle cell anemia, sarcoid, pseudoxanthoma elasticum, Pagets disease, vein occlusion, artery occulsion, carotid obstructive disease, chronic uveitis, chronic vitritis, Lyme's disease, Eales disease, Bechets disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, post-laser complications, abnormal proliferation of fibrovascular tissue, hemangiomas, Osler-Weber-Rendu, acquired immune deficiency syndrome, ocular neovascular disease, osteoarthritis, chronic inflammation, Crohn's disease, ulceritive colitis, psoriasis., atherosclerosis, and pemphigoid. See U.S. Patent No. 5,712,291)

The invention is also broadly applicable to methods for inhibiting tumor growth and metastasis, reduction of scar tissue formation, reduction of complications due to cell adhesion in organ transplants, and the inhibition of lymphocyte adhesion and mobility.

While the fundamental novel features of the invention have been shown and described, it will be understood that various omissions, substitutions, and changes in the form and details illustrated may be made by those skilled in the art without departing from the spirit of the invention. For example, various modifications,

additions, and/or substitutions can be made to the type IV collagen α monomer chains that would be encompassed by the invention.

We claim

1. A method for inhibiting angiogenesis in an animal tissue comprising contacting the tumor or animal tissue with an amount effective to inhibit angiogenesis of a polypeptide composition comprising one or more non-RGD containing integrin $\alpha v \beta 3$ antagonists.
5
2. A method for inhibiting angiogenesis in an animal tissue comprising contacting the tumor or animal tissue with an amount effective to inhibit angiogenesis of a polypeptide composition comprising one or more antagonists of $\alpha v \beta 5$ integrin.
3. A method for inhibiting angiogenesis in an animal tissue comprising contacting
10 the tumor or animal tissue with an amount effective to inhibit angiogenesis of a polypeptide composition comprising one or more antagonists of $\beta 1$ integrins.
4. A method for inhibiting angiogenesis in an animal tissue comprising contacting the tumor or animal tissue with an amount effective to inhibit angiogenesis of a polypeptide composition comprising one or more antagonists of $\alpha 3 \beta 1$ integrin.
- 15 5. A method for inhibiting endothelial cell adhesion to extracellular matrix, comprising contacting the endothelial cell with an amount effective to inhibit endothelial cell adhesion to extracellular matrix of a polypeptide composition comprising one or more non-RGD containing integrin $\alpha v \beta 3$ antagonists.
6. A method for inhibiting endothelial cell adhesion to extracellular matrix,
20 comprising contacting the endothelial cell with an amount effective to inhibit endothelial cell adhesion to extracellular matrix of a polypeptide composition comprising one or more antagonists of $\alpha v \beta 5$ integrin.
7. A method for inhibiting endothelial cell adhesion to extracellular matrix, comprising contacting the endothelial cell with an amount effective to inhibit endothelial

cell adhesion to extracellular matrix of a polypeptide composition comprising one or more antagonists of $\alpha 3\beta 1$ integrin.

8. A method for inhibiting endothelial cell adhesion to extracellular matrix, comprising contacting the endothelial cell with an amount effective to inhibit endothelial cell adhesion to extracellular matrix of a polypeptide composition comprising one or more antagonists of $\beta 1$ integrins.

9. A method for inhibiting tumor metastasis in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor metastasis of a polypeptide composition comprising one or more non-RGD containing integrin $\alpha v\beta 3$ antagonists.

10. A method for inhibiting tumor metastasis in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor metastasis of a polypeptide composition comprising one or more antagonists of $\alpha v\beta 5$ integrin.

11. A method for inhibiting tumor metastasis in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor metastasis of a polypeptide composition comprising one or more antagonists of $\alpha 3\beta 1$ integrin.

12. A method for inhibiting tumor metastasis in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor metastasis of a polypeptide composition comprising one or more antagonists of $\beta 1$ integrins.

13. A method for inhibiting tumor growth in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor growth of a polypeptide composition comprising one or more non-RGD containing integrin $\alpha v\beta 3$ antagonists.

14. A method for inhibiting tumor growth in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor growth of a polypeptide composition comprising one or more antagonists of $\alpha v\beta 5$ integrin.

15. A method for inhibiting tumor growth in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor growth of a polypeptide composition comprising one or more antagonists of $\alpha 3 \beta 1$ integrin.
16. A method for inhibiting tumor growth in tissue comprising contacting the tumor
5 or tissue with an amount effective to inhibit tumor growth of a polypeptide composition comprising one or more antagonists of $\beta 1$ integrins.

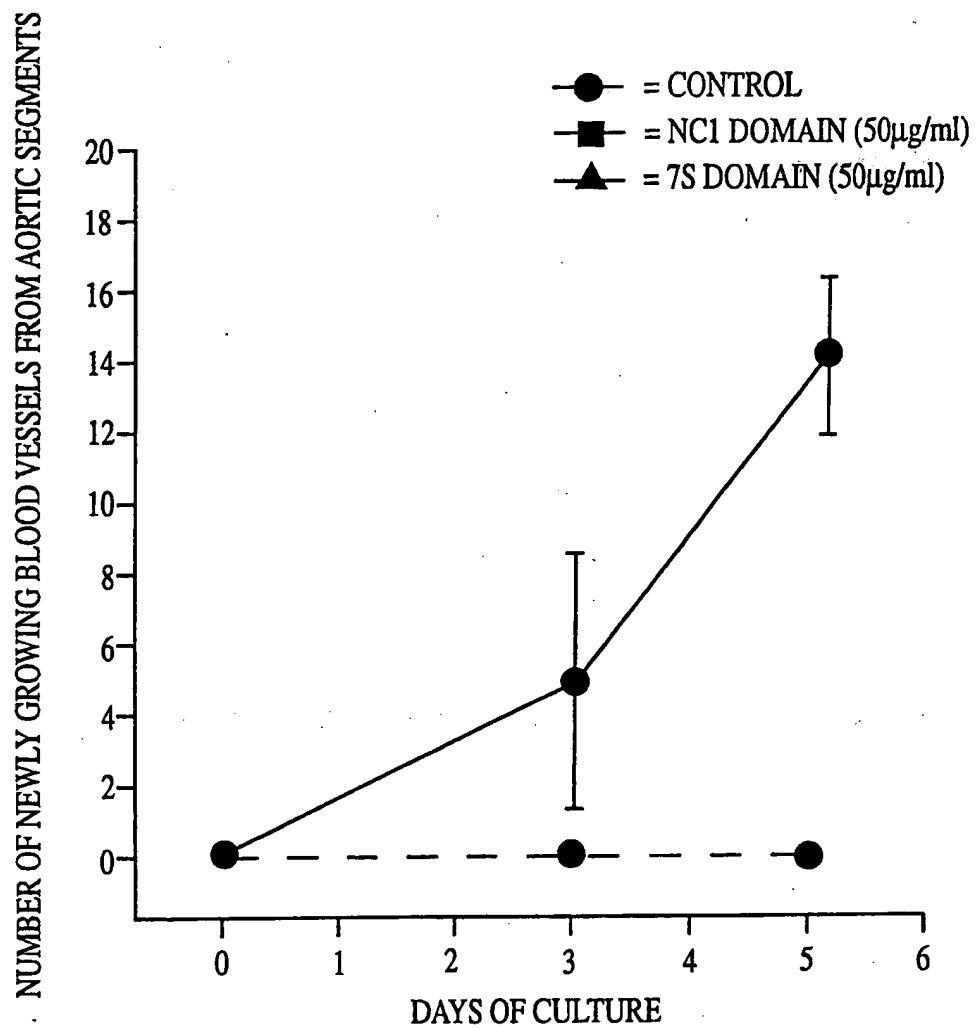


FIG. 1

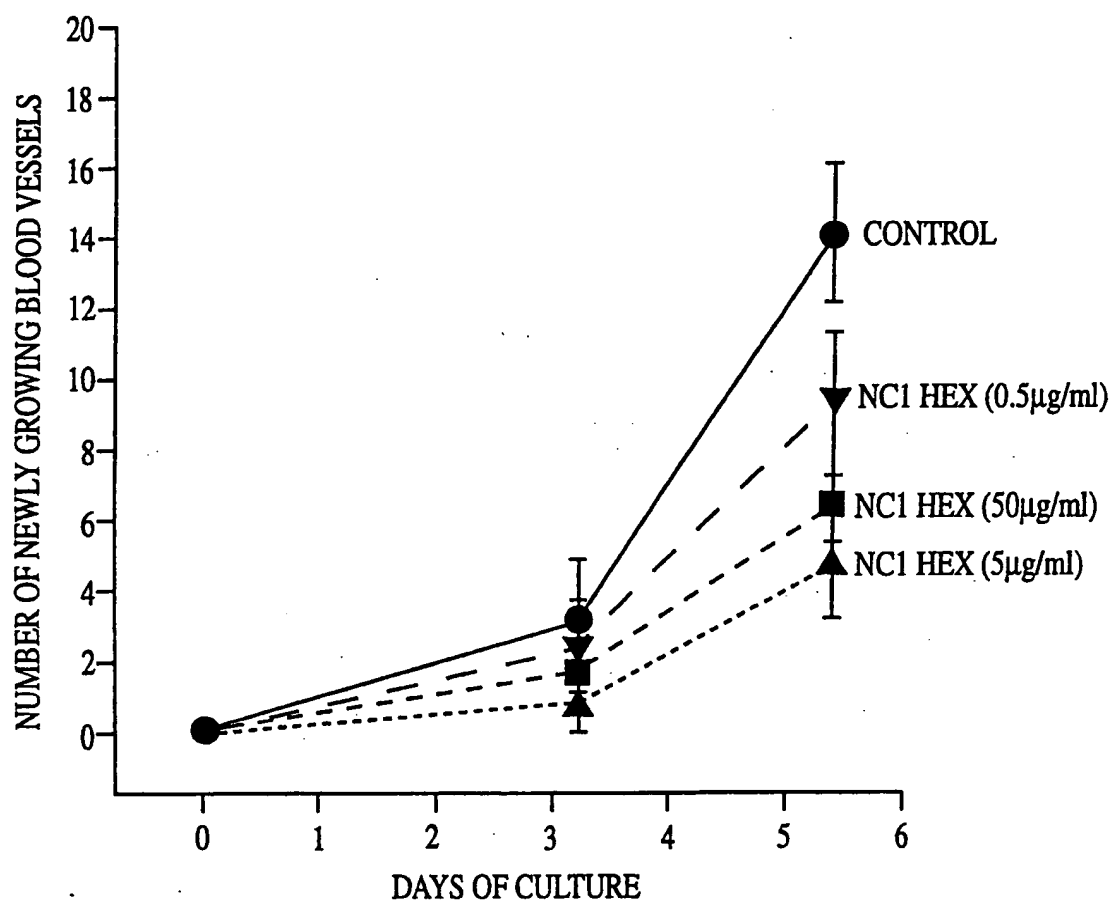


FIG. 2

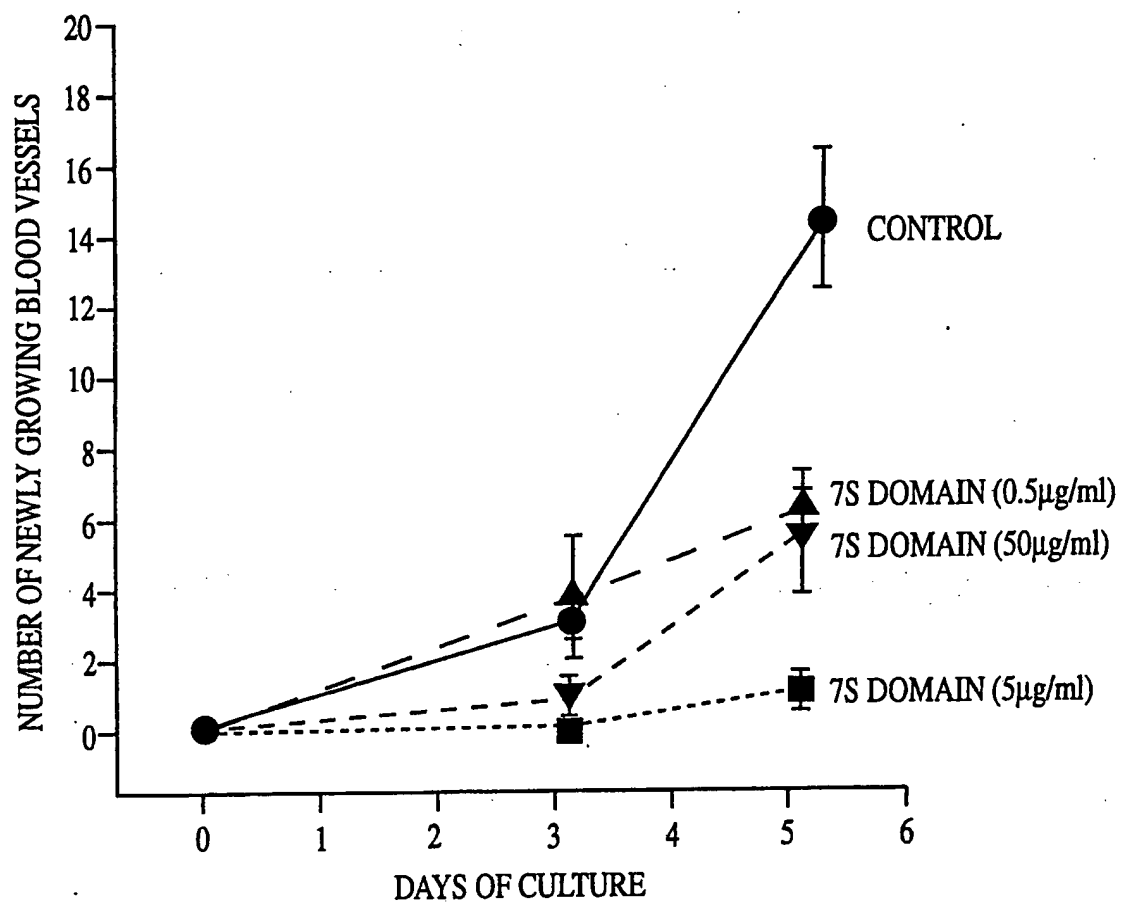


FIG. 3



CONTROL
FIG. 4a



7S DOMAIN (50 μ g/ml)
FIG. 4b



NC1 DOMAIN (50 μ g/ml)
FIG. 4c

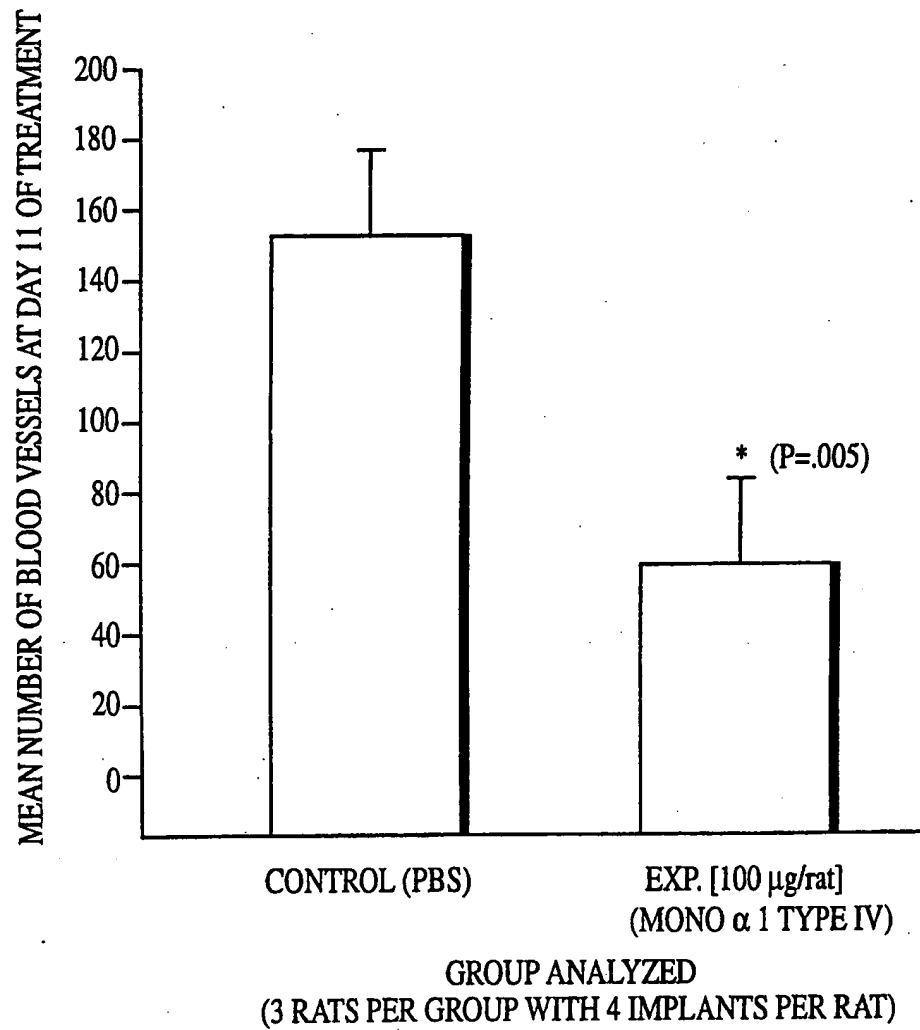


FIG. 5

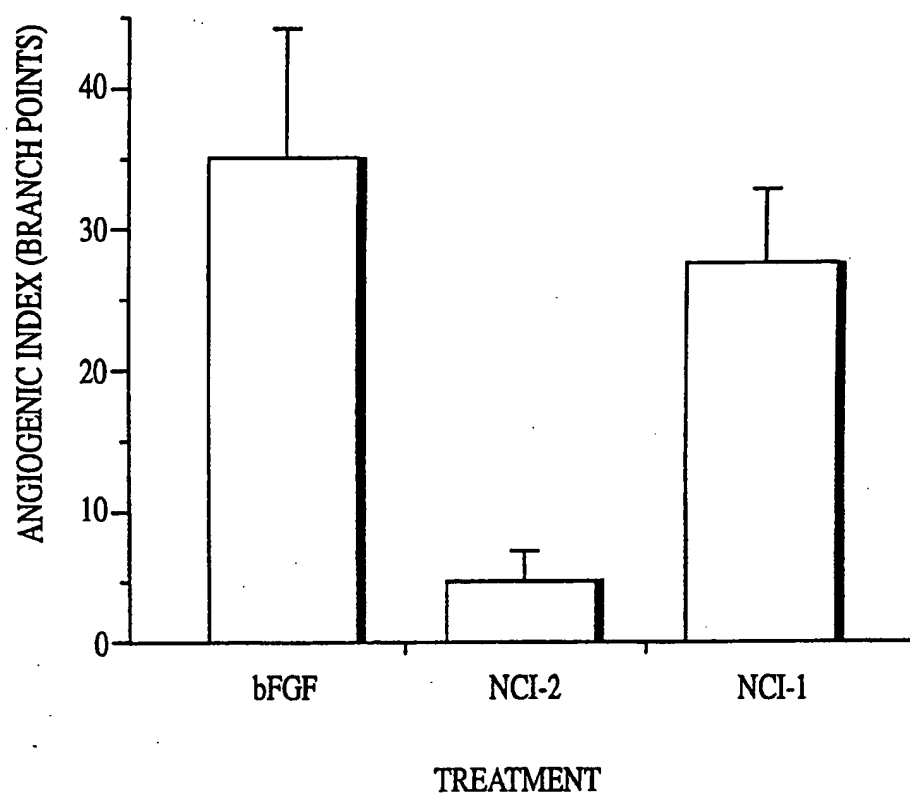


FIG. 6

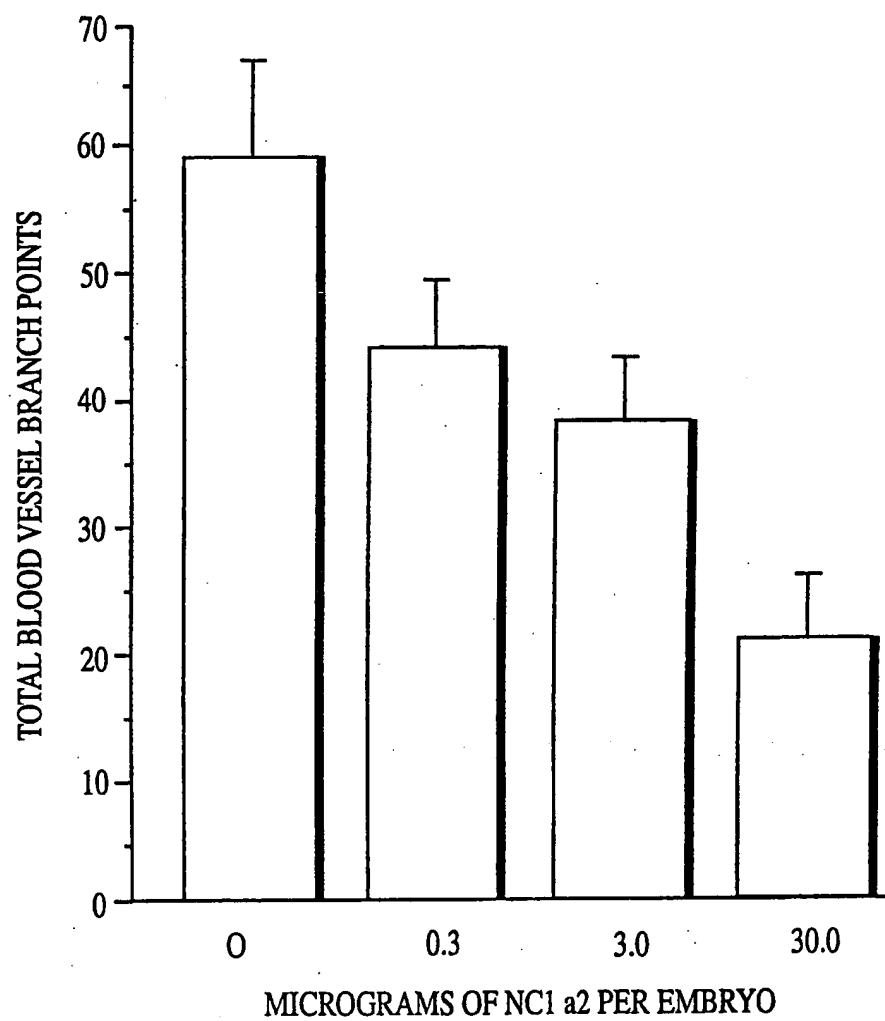


FIG. 7

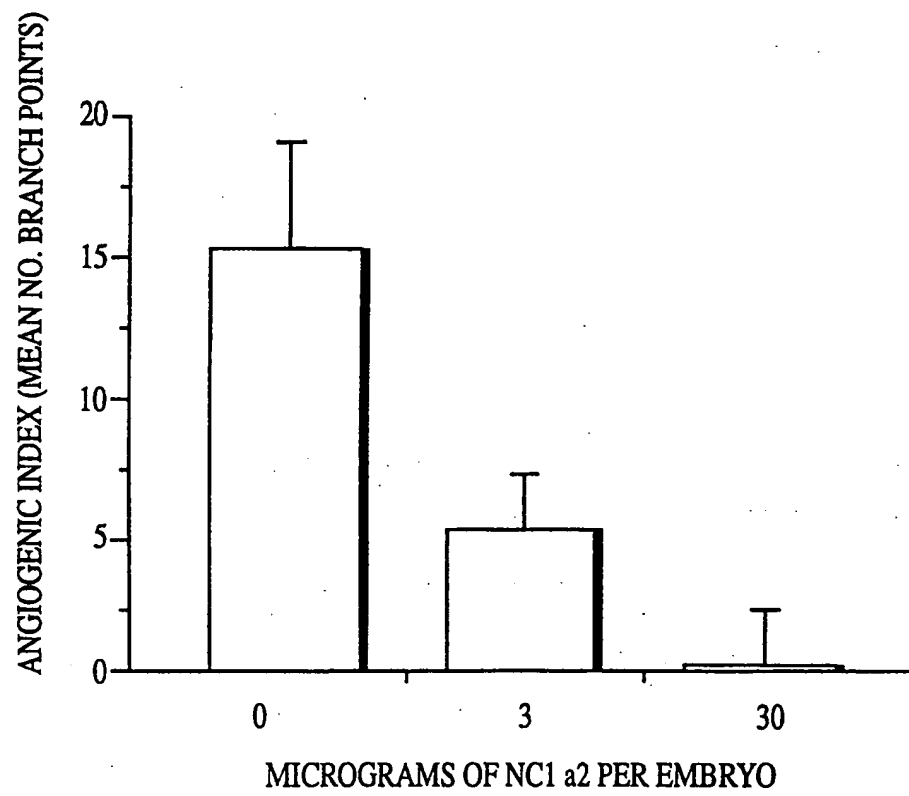


FIG. 8

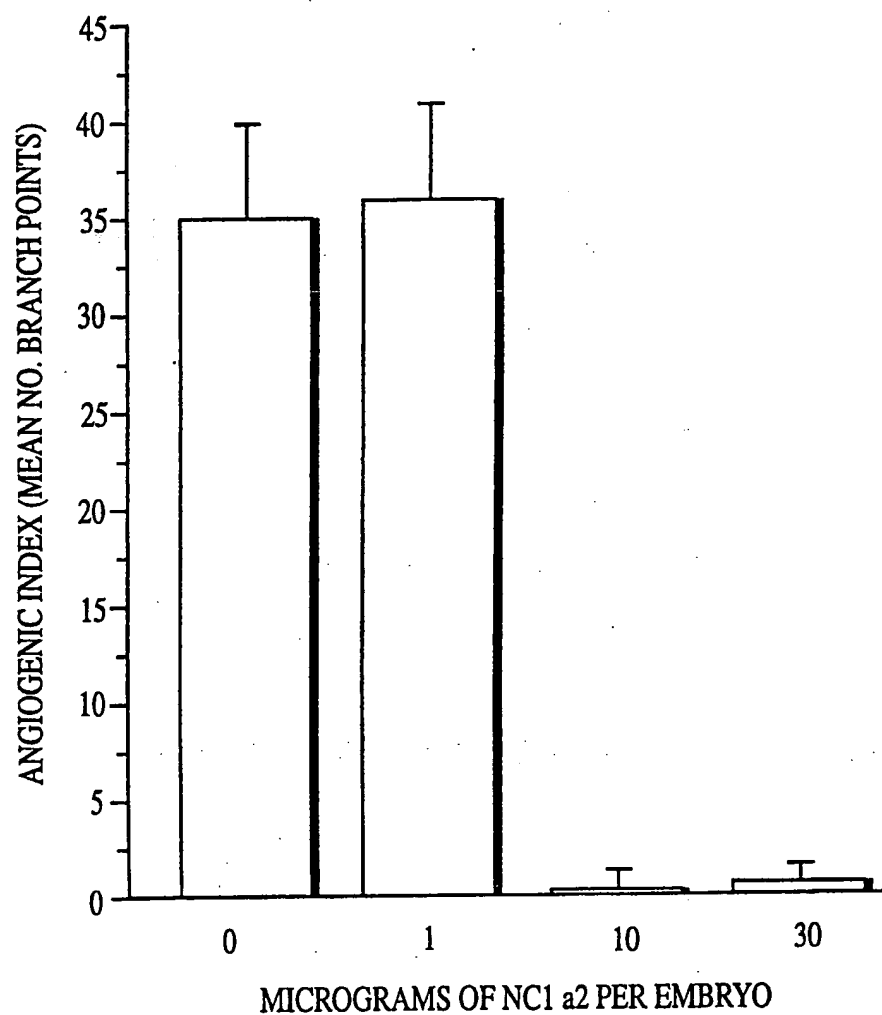


FIG. 9

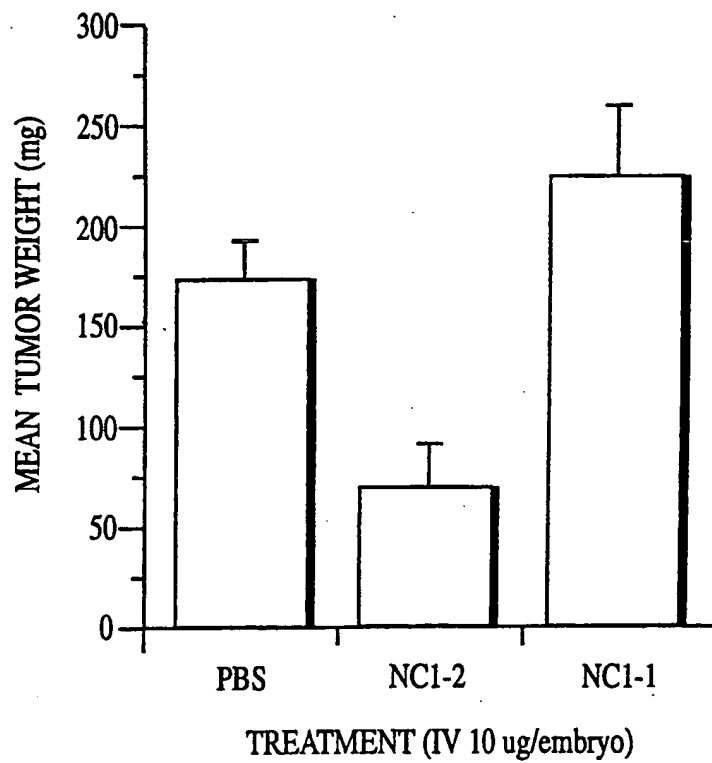


FIG. 10

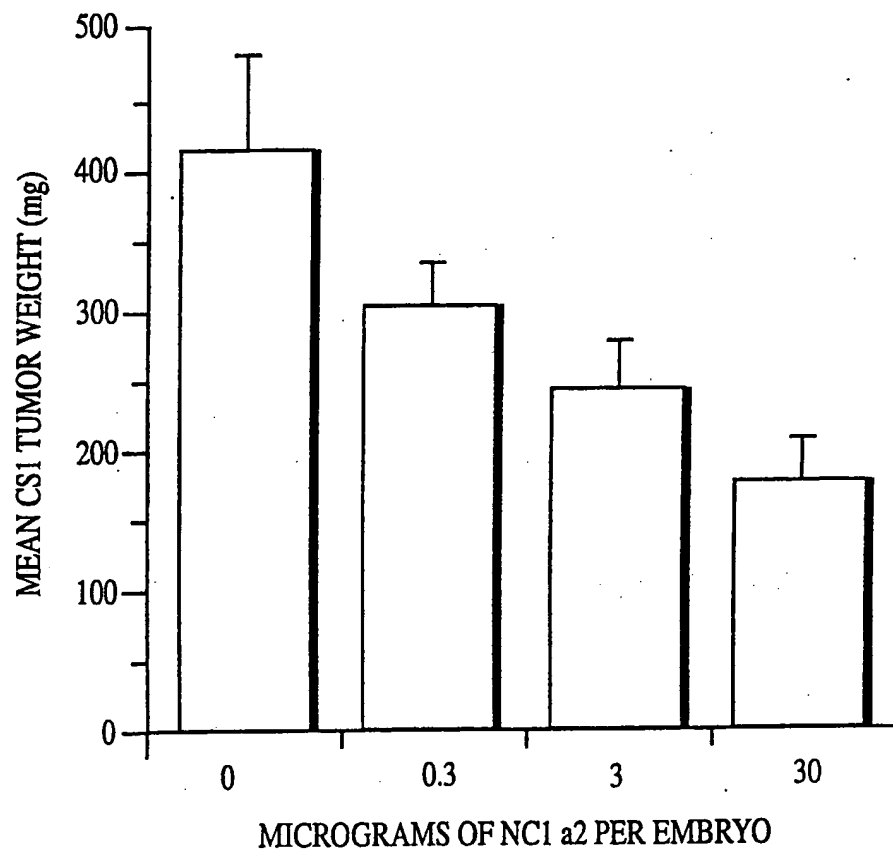


FIG. 11

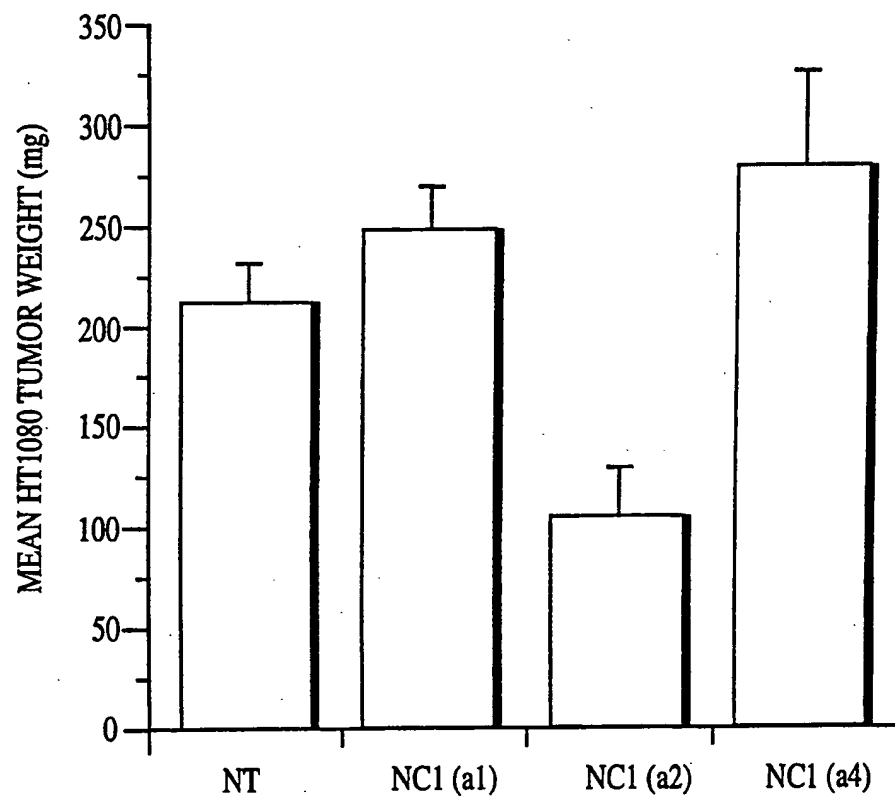


FIG. 12

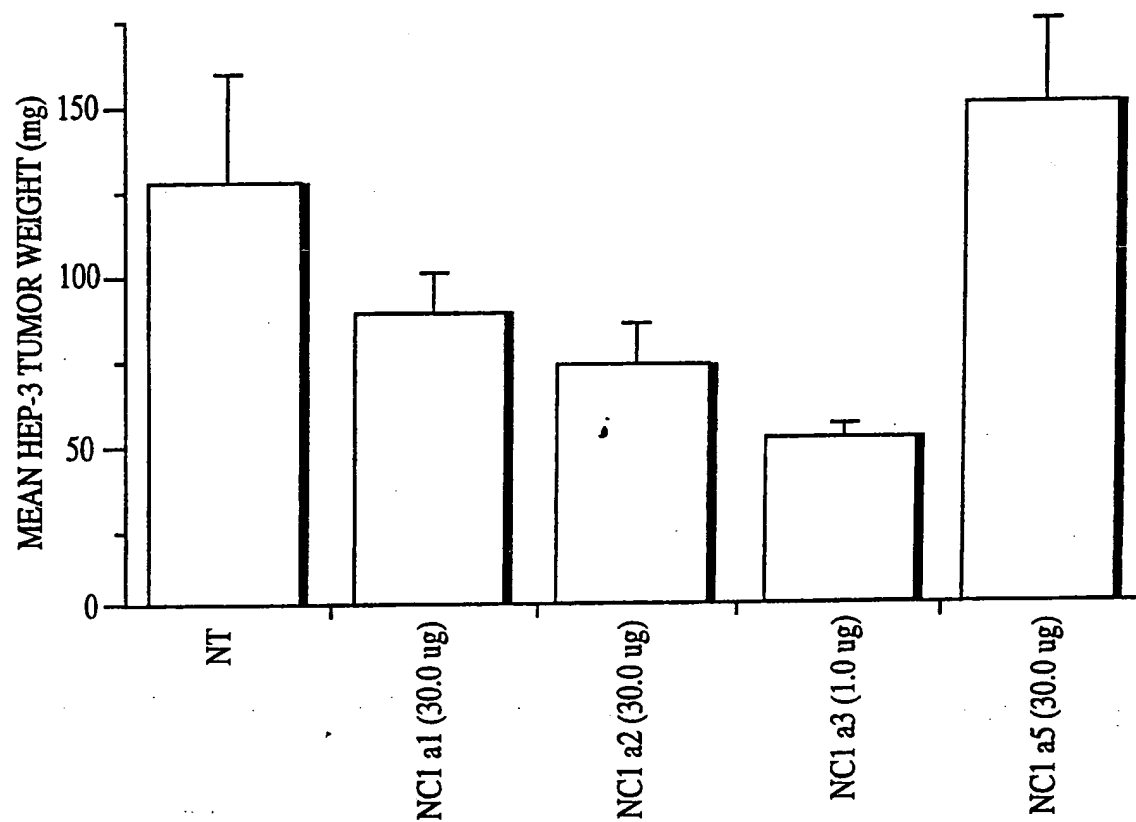


FIG. 13

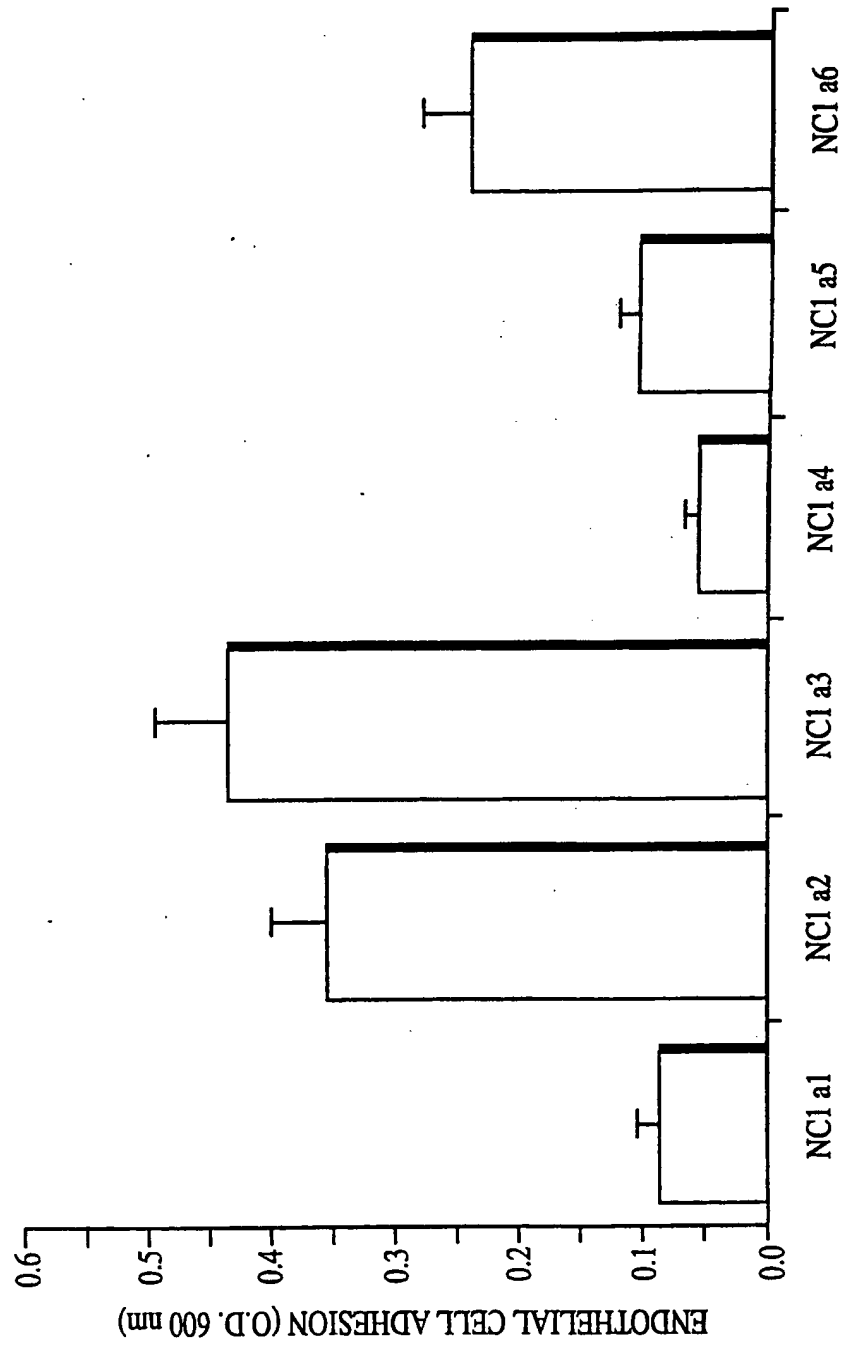


FIG. 14

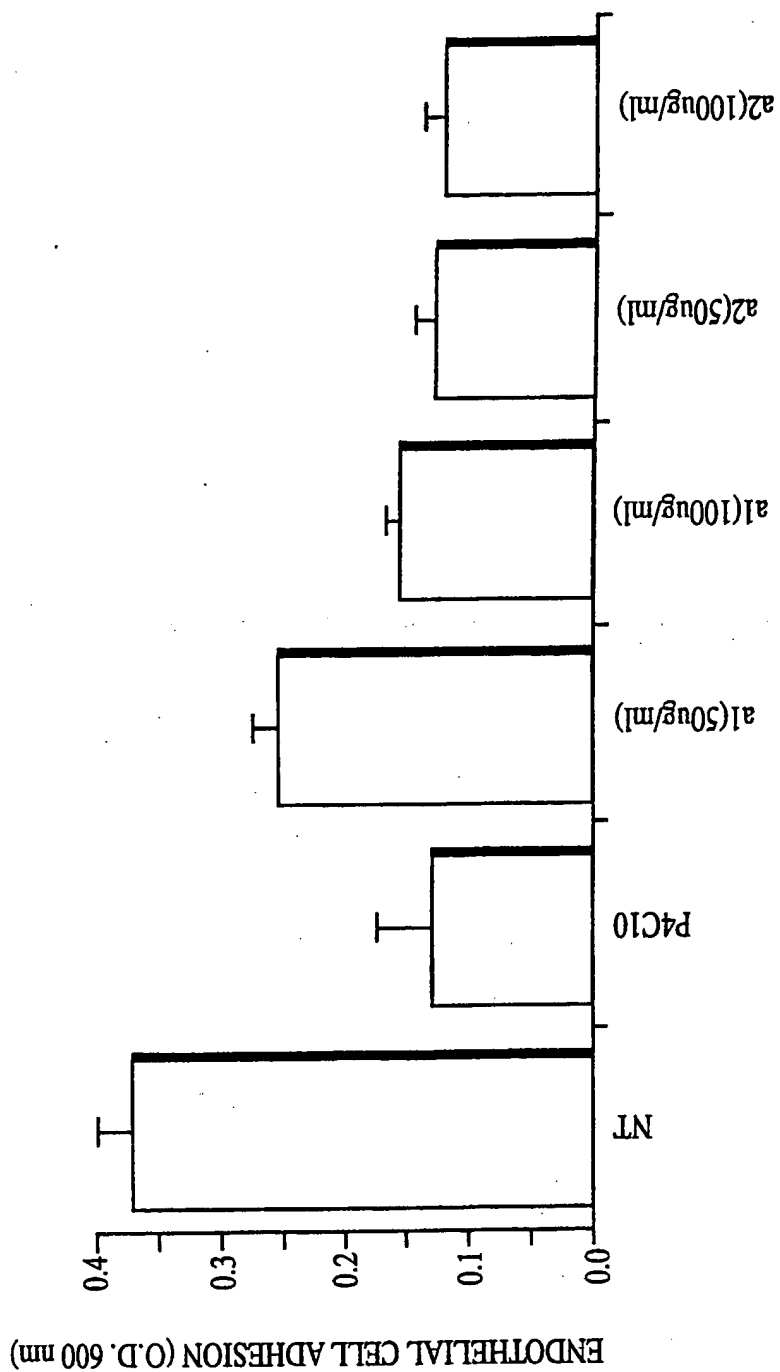


FIG. 15

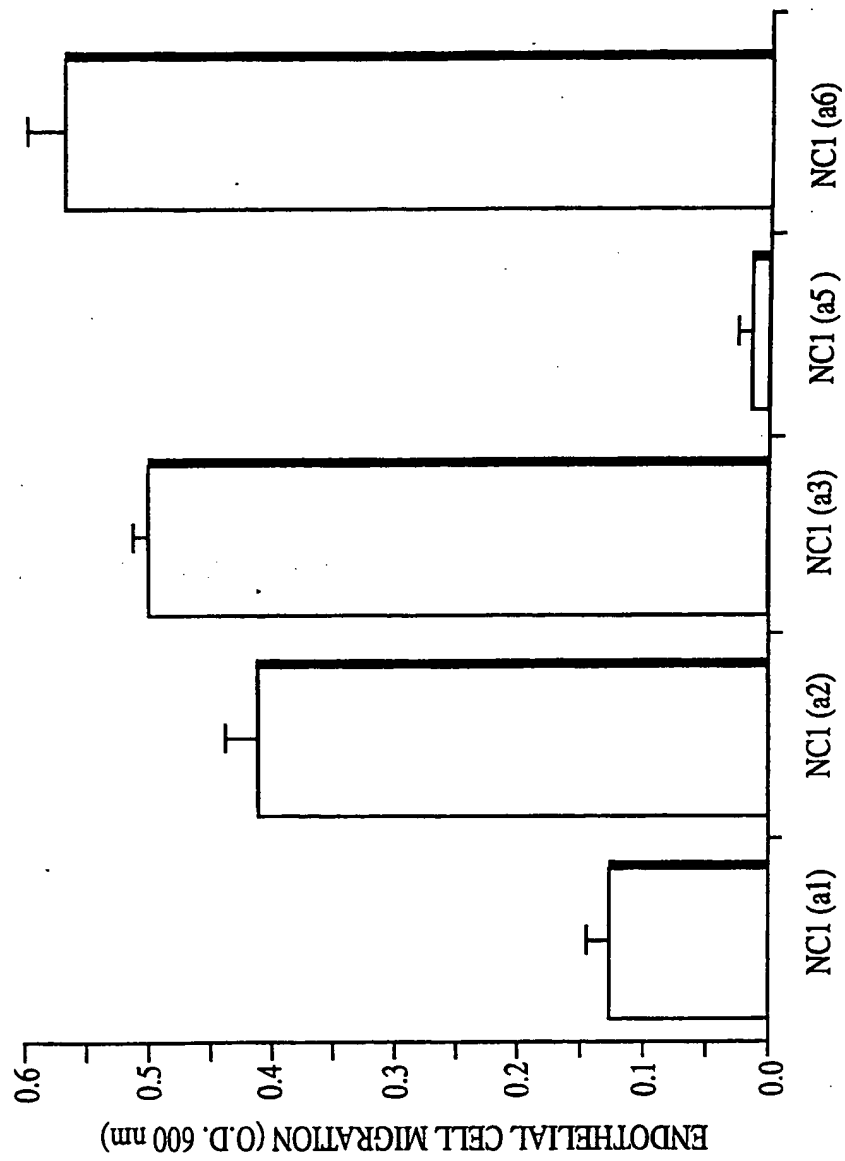


FIG. 16

A. $\alpha 1$ (IV) NC1

900 910 920 930 940 950
| | | | |
CTGCCGCCTGCCTGCCTGCCACTGAGGGTTCCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCACTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | |
GACAAGCTAGCATCTGTTGATCACGGCTTCCTTGTGACCAGGCATAGTCAAACAATAGAT
D K L A S V D H G F L V T R H S Q T I D

1080 1090 1100 1110 1120 1130
| | | | |
GACCCACAGTGTCTTCTGGGACCAAATCTTTACCACGGGTACTCTTTGCTCTACGTG
D P Q C P S G T K I L Y H G Y S L L Y V

1140 1150 1160 1170 1180 1190
| | | | |
CAAGGCAATGAACGGGCCCCATGGCCAGGACTTGGGCACGGCCGGCAGCTGCCTGCGCAAG
Q G N E R A H G Q D L G T A G S C L R K

1200 1210 1220 1230 1240 1250
| | | | |
TTCAGCACAAATGCCCTTCCTGTTCTGCAATATTAACAACGTGTGCAACTTTGCATCACGA
F S T M P F L F C N I N N V C N F A S R

1260 1270 1280 1290 1300 1310
| | | | |
AATGACTACTCGTACTGGCTGTCCACCCCTGAGCCCATGCCCATGTCAATGGCACCCATC
N D Y S Y W L S T P E P M P M S M A P I

1320 1330 1340 1350 1360 1370
| | | | |
ACGGGGGAAAACATAAGACCATTATTAGTAGGTGTGCTGTGTGTGAGGCGCCTGCCATG
T G E N I R P F I S R C A V C E A P A M

1380 1390 1400 1410 1420 1430
| | | | |
GTGATGGCCGTGCACAGCCAGACCATTGATCCACCGTGCCCCAGCGGGTGGTCCTCG
V M A V H S Q T I Q I P P C P S G W S S

1440 1450 1460 1470 1480 1490
| | | | |
CTGTGGATCGGCTACTCTTTGTGATGCACACCAGCGCTGGTGCAGAAGGCTCTGGCCAA
L W I G Y S F V M H T S A G A E G S G Q

1500 1510 1520 1530 1540 1550
| | | | |
GCCCTGGCGTCCCCCGGCTCCTGCCTGGAGGAGTTTAGAAGTGCGCCATTTCATCGAGTGT

FIG. 17a

A L A S P G S C L E E F R S A P F I E C

1560 1570 1580 1590 1600 1610
CACGGCCGTGGGACCTGCAATTACTACGCAAACGCTTACAGCTTTTGGCTCGCCACCATA
H G R G T C N Y Y A N A Y S F W L A T I

1620 1630 1640 1650 1660 1670
GAGAGGAGCGAGATGTTCAAGAAGCCTACGCCGTCCACCTTGAAGGCAGGGGAGCTGCGC
E R S E M F K K P T P S T L K A G E L R

1680 1690 1700 1710 1720 1730
ACGCACGTCAGCCGCTGCCAAGTCTGTATGAGAAGAACATAATGAAGCCTGACTCAGCTA
T H V S R C Q V C M R R T - -

1740 1750 1760 1770 1780 1790
CCGCGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTG

FIG. 17a

B. $\alpha 2$ (IV) NC1

900 910 920 930 940 950
| | | | | |
CTGCCGCCTGCCTGCCACTGAGGGTCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCACTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | | |
GACAAGCTAGCCGTCAGCATCGGCTACCTCCTGGTGAAGCACAGCCAGACGGACCAGGAG
D K L A V S I G Y L L V K H S Q T D Q E

1080 1090 1100 1110 1120 1130
| | | | | |
CCCATGTGCCCGGTGGGCATGAACAACTCTGGAGTGGATACAGCCTGCTGTACTTCCGAG
P M C P V G M N K L W S G Y S L L Y F E

1140 1150 1160 1170 1180 1190
| | | | | |
GGCCAGGAGAAGGCGCACAACCAGGACCTGGGGCTGGCGGGCTCCTGCCTGGCGCGGTTC
G Q E K A H N Q D L G L A G S C L A R F

1200 1210 1220 1230 1240 1250
| | | | | |
AGCACCATGCCCTTCCTGTACTGCAACCCTGGTGATGTCTGCTACTATGCCAGCCGGAAC
S T M P F L Y C N P G D V C Y Y A S R N

1260 1270 1280 1290 1300 1310
| | | | | |
GACAAGTCCTACTGGCTCTCTACCACTGCGCCGCTGCCCATGATGCCCGTGGCCGAGGAC
D K S Y W L S T T A P L P M M P V A E D

1320 1330 1340 1350 1360 1370
| | | | | |
GAGATCAAGCCCTACATCAGCCGCTGTTCTGTGTGTGAGGCCCCGGCCATCGCCATCGCG
E I K P Y I S R C S V C E A P A I A I A

1380 1390 1400 1410 1420 1430
| | | | | |
GTCCACAGTCAGGATGTCTCCATCCACACTGCCCAGCTGGGTGGCGGAGTTTGTGGATC
V H S Q D V S I P H C P A G W R S L W I

1440 1450 1460 1470 1480 1490
| | | | | |
GGATATTCCTTCCTCATGCACACGGCGCGGAGACGAAGCGGTGGCCAATCACTGGTG
G Y S F L M H T A A G D E G G G Q S L V

1500 1510 1520 1530 1540 1550

FIG. 17b

TCACCGGGCAGCTGTCTAGAGGACTTCCGCGCCACACCATTTCATCGAATGCAATGGAGGG
S P G S C L E D F R A T P F I E C N G G

1560 1570 1580 1590 1600 1610
CGCGGCACCTGCCACTACTACGCCAACAAGTACAGCTTCTGGCTGACCACCATTCCCGAG
R G T C H Y Y A N K Y S F W L T T I P E

1620 1630 1640 1650 1660 1670
CAGAGCTTCCAGGGCTCGCCCTCCGCCGACACGCTCAAGGCCGGCCTCATCCGCACACAC
Q S F Q G S P S A D T L K A G L I R T H

1680 1690 1700 1710 1720 1730
ATCAGCCGCTGCCAGGTGTGCATGAAGAACCTGTGAGCCGGCGCGTGCCAGGGCCCTATT
I S R C Q V C M K N L -

1740 1750 1760 1770 1780 1790
CTATAGTGTACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGC

FIG. 17b

C. $\alpha 3$ (IV)NC1

900 910 920 930 940 950
CTGCCGCCTGCCTGCCACTGAGGGTTCACAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCGCTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
GACAAACGTGGAGACAGTGGATCACCTGCAACCTGGACAACGAGAGGCTTTGTCTTCACC
D K R G D S G S P A T W T T R G F V F T

1080 1090 1100 1110 1120 1130
CGACACAGTCAAACCACAGCAATTCCTTCATGTTCAGAGGGGACAGTGCCACTCTACAGT
R H S Q T T A I P S C P E G T V P L Y S

1140 1150 1160 1170 1180 1190
GGGTTTCTTTTCTTTTGTACAAGGAAATCAACGAGCCACGGACAAGACCTTGGAAC
G F S F L F V Q G N Q R A H G Q D L G T

1200 1210 1220 1230 1240 1250
CTTGGCAGCTGCCTGCAGCGATTACCACAATGCCATTCTTATTCTGCAATGTCAATGAT
L G S C L Q R F T T M P F L F C N V N D

1260 1270 1280 1290 1300 1310
GTATGTAATTTGCATCTCGAAATGATTATTCACTGGCTGTCAACACCAGCTCTGATG
V C N F A S R N D Y S Y W L S T P A L M

1320 1330 1340 1350 1360 1370
CCAATGAACATGGCTCCCATTACTGGCAGAGCCCTTGAGCCTTATATAAGCAGATGCACT
P M N M A P I T G R A L E P Y I S R C T

1380 1390 1400 1410 1420 1430
GTTTGTGAAGGTCCTGCGATCGCCATAGCCGTTACAGCCAAACCACTGACATTCTCCCA
V C E G P A I A I A V H S Q T T D I P P

1440 1450 1460 1470 1480 1490
TGTCTCACGGCTGGATTCTCTCTGGAAAGGATTTTCATTTCATCATGTTTACAAGTGCA
C P H G W I S L W K G F S F I M F T S A

1500 1510 1520 1530 1540 1550

FIG. 17c

```
      |      |      |      |      |      |
GGTTCTGAGGGCGCCGGGCAAGCACTGGCCTCCCCCGGCTCCTGCCTGGAAGAATTCCGA
G S E G A G Q A L A S P G S C L E E F R

1560      1570      1580      1590      1600      1610
      |      |      |      |      |      |
GCCAGCCCATTTCTAGAATGTCATGGAAGAGGAACGTGCAACTACTATTCAAATTCCTAC
A S P F L E C H G R G T C N Y Y S N S Y

1620      1630      1640      1650      1660      1670
      |      |      |      |      |      |
AGTTTCTGGCTGGCTTCATTAAACCCAGAAAGAATGTTTCAGAAAGCCTATTCCATCAACT
S F W L A S L N P E R M F R K P I P S T

1680      1690      1700      1710      1720      1730
      |      |      |      |      |      |
GTGAAAGCTGGGGAATTAGAAAAAATAATAAGTCGCTGTCAGGTGTGCATGAAGAAAAGA
V K A G E L E K I I S R C Q V C M K K R

1740      1750      1760      1770      1780      1790
      |      |      |      |      |      |
CACTGAGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGCTGATCAGCCTCGAC

H -
```

FIG. 17c

D. $\alpha 4$ (IV)NC1

900 910 920 930 940 950
| | | | | |
CTGCCGCCTGCCTGCCTGCCACTGAGGGTCCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCGCTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | | |
GACAAGCCTGGATACCTCGGTGGCTTCTCTCTGGTTCTCCACAGTCAGACGGACCAGGAG
D K P G Y L G G F L L V L H S Q T D Q E

1080 1090 1100 1110 1120 1130
| | | | | |
CCCACCTGCCCCCTGGGCATGCCAGGCTCTGGACTGGGTATAGTCTGTTATACCTGGAA
P T C P L G M P R L W T G Y S L L Y L E

1140 1150 1160 1170 1180 1190
| | | | | |
GGGCAAGAGAAAGCTCACAATCAAGACCTTGGTCTGGCAGGGTCTTGCCTTCCCGTATTT
G Q E K A H N Q D L G L A G S C L P V F

1200 1210 1220 1230 1240 1250
| | | | | |
AGCACGCTGCCCTTTGCCTACTGCAACATCCACCAGGTGTGCCACTATGCCCAGAGAAAC
S T L P F A Y C N I H Q V C H Y A Q R N

1260 1270 1280 1290 1300 1310
| | | | | |
GACAGATCCTACTGGCTGGCCAGCGCTGCGCCCCCTCCCCATGATGCCACTCTCTGAAGAG
D R S Y W L A S A A P L P M M P L S E E

1320 1330 1340 1350 1360 1370
| | | | | |
GCGATCCGCCCCTATGTCAGCCGCTGTGCGGTATGCGAGGCCCCGGCCCAGGCGGTGGCG
A I R P Y V S R C A V C E A P A Q A V A

1380 1390 1400 1410 1420 1430
| | | | | |
GTGCACAGCCAGGACCAGTCCATCCCCCATGTCCGCAGACCTGGAGGAGCCTCTGGATC
V H S Q D Q S I P P C P Q T W R S L W I

1440 1450 1460 1470 1480 1490
| | | | | |
GGGTATTCATTCTGATGCACACAGGAGCTGGGGACCAAGGAGGAGGGCAGGCCCTTATG
G Y S F L M H T G A G D Q G G G Q A L M

1500 1510 1520 1530 1540 1550

FIG. 17d

TCACCTGGCAGCTGCCTGGAAGATTTTCAGAGCAGCACCATTCTTGAATGCCAGGGCCGG
S P G S C L E D F R A A P F L E C Q G R

1560 1570 1580 1590 1600 1610
CAGGGAACCTTGCCACTTTTTTCGCAAATAAGTATAGCTTCTGGCTCACAACGGTGAAAGCA
Q G T C H F F A N K Y S F W L T T V K A

1620 1630 1640 1650 1660 1670
GACTTGCACTTTTCCTCTGCTCCAGCACCAGACACCTTAAAAGAAAGCCAGGCCCAACGC
D L Q F S S A P A P D T L K E S Q A Q R

1680 1690 1700 1710 1720 1730
CAGAAAATCAGCCGGTGCCAGGTCTGCGTGAAGTATAGCTAGGGGCCCTATTCTATAGTG
Q K I S R C Q V C V K Y S -

1740 1750 1760 1770 1780 1790
TCACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATC

FIG. 17d

E. $\alpha 5$ (IV) NC1

900 910 920 930 940 950
CTGCCGCCTGCCTGCCACTGAGGGTTCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCGCTAGCTGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
GACAAAGGTCCCCCTGGAACCTCCTCTGTTGCACATGGATTCTTATTACACGCCACAGC
D K G P P G T S S V A H G F L I T R H S

1080 1090 1100 1110 1120 1130
CAGACAACGGATGCACCACAATGCCCACAGGGAACACTTCAGGTCTATGAAGGCTTTTCT
Q T T D A P Q C P Q G T L Q V Y E G F S

1140 1150 1160 1170 1180 1190
CTCCTGTATGTACAAGGAAATAAAAGAGCCCACGGTCAAGACTTGGGGACGGCTGGCAGC
L L Y V Q G N K R A H G Q D L G T A G S

1200 1210 1220 1230 1240 1250
TGCCTTCGTGCTTTAGTACCATGCCTTTCATGTTCTGCAACATCAATAATGTTTGCAAC
C L R R F S T M P F M F C N I N N V C N

1260 1270 1280 1290 1300 1310
TTTGCTTCAAGAAATGACTATTCTTACTGGCTCTCTACCCAGAGCCCATGCCAATGAGC
F A S R N D Y S Y W L S T P E P M P M S

1320 1330 1340 1350 1360 1370
ATGCAACCCCTAAAGGGCCAGAGCATCCAGCCATTCATTAGTCGATGTGCAGTATGTGAA
M Q P L K G Q S I Q P F I S R C A V C E

1380 1390 1400 1410 1420 1430
GCTCCAGCTGTGGTGATCGCAGTTTCACAGTCAGACGATCCAGATTCCCCATTGTCCTCAG
A P A V V I A V H S Q T I Q I P H C P Q

1440 1450 1460 1470 1480 1490
GGATGGGATTCTCTGTGGATTGGTTATTCTTCATGATGCATACAAGTGCAGGGGCAGAA
G W D S L W I G Y S F M M H T S A G A E

1500 1510 1520 1530 1540 1550

FIG. 17e

GGCTCAGGTCAAGCCCTAGCCTCCCCTGGTTCCTGCTTGAAGAGTTTCGTTTCAGCTCCC
G S G Q A L A S P G S C L E E F R S A P

1560 1570 1580 1590 1600 1610
TTCATCGAATGTCATGGGAGGGGTACCTGTAACCTACTATGCCAACTCCTACAGCTTTTGG
F I E C H G R G T C N Y Y A N S Y S F W

1620 1630 1640 1650 1660 1670
CTGGCAACTGTAGATGTGTCTAGACATGTTTCAGTAAACCTCAGTCAGAAACGCTGAAAGCA
L A T V D V S D M F S K P Q S E T L K A

1680 1690 1700 1710 1720 1730
GGAGACTTGAGGACACGAATTAGCCGATGTCAAGTGTGCATGAAGAGGACATAACGCGGC
G D L R T R I S R C Q V C M K R T -

1740 1750 1760 1770 1780 1790
CGCTCGAGCATGCATCTAGAGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGC

FIG. 17e.

F. $\alpha 6$ (IV) NCL

900 910 920 930 940 950
| | | | | |
CTGCCGCCTGCCTGCCTGCCACTGAGGGTTCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCACTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | | |
GACAAGCTAGCGAGCATGAGAGTGGGCTACACGTTGGTAAAGCACAGCCAGTCGGAACAG
D K L A S M R V G Y T L V K H S Q S E Q

1080 1090 1100 1110 1120 1130
| | | | | |
GTGCCCCCGTGTCCCATCGGGATGAGCCAGCTGTTGGGTGGGTACAGCTTACTGTTTGTG
V P P C P I G M S Q L W V G Y S L L F V

1140 1150 1160 1170 1180 1190
| | | | | |
GAGGGGCAAGAGAAAGCCCACAACCAGGACCTGGGCTTTGCTGGCTCCTGTCTGCCCCGC
E G Q E K A H N Q D L G F A G S C L P R

1200 1210 1220 1230 1240 1250
| | | | | |
TTCAGCACCATGCCCTTCATCTACTGCAACATCAACGAGGTGTGCCACTATGCCAGGCGC
F S T M P F I Y C N I N E V C H Y A R R

1260 1270 1280 1290 1300 1310
| | | | | |
AATGATAAATCTTACTGGCTCTCCACTACCGCCCCCTATCCCCATGATGCCCGTCAGCCAG
N D K S Y W L S T T A P I P M M P V S Q

1320 1330 1340 1350 1360 1370
| | | | | |
ACCCAGATTCCCCAGTACATCAGCCGCTGCTCTGTGTGTGAGGCACCCTCGCAAGCCATT
T Q I P Q Y I S R C S V C E A P S Q A I

1380 1390 1400 1410 1420 1430
| | | | | |
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A V H S Q D I T I P Q C P L G W R S L W

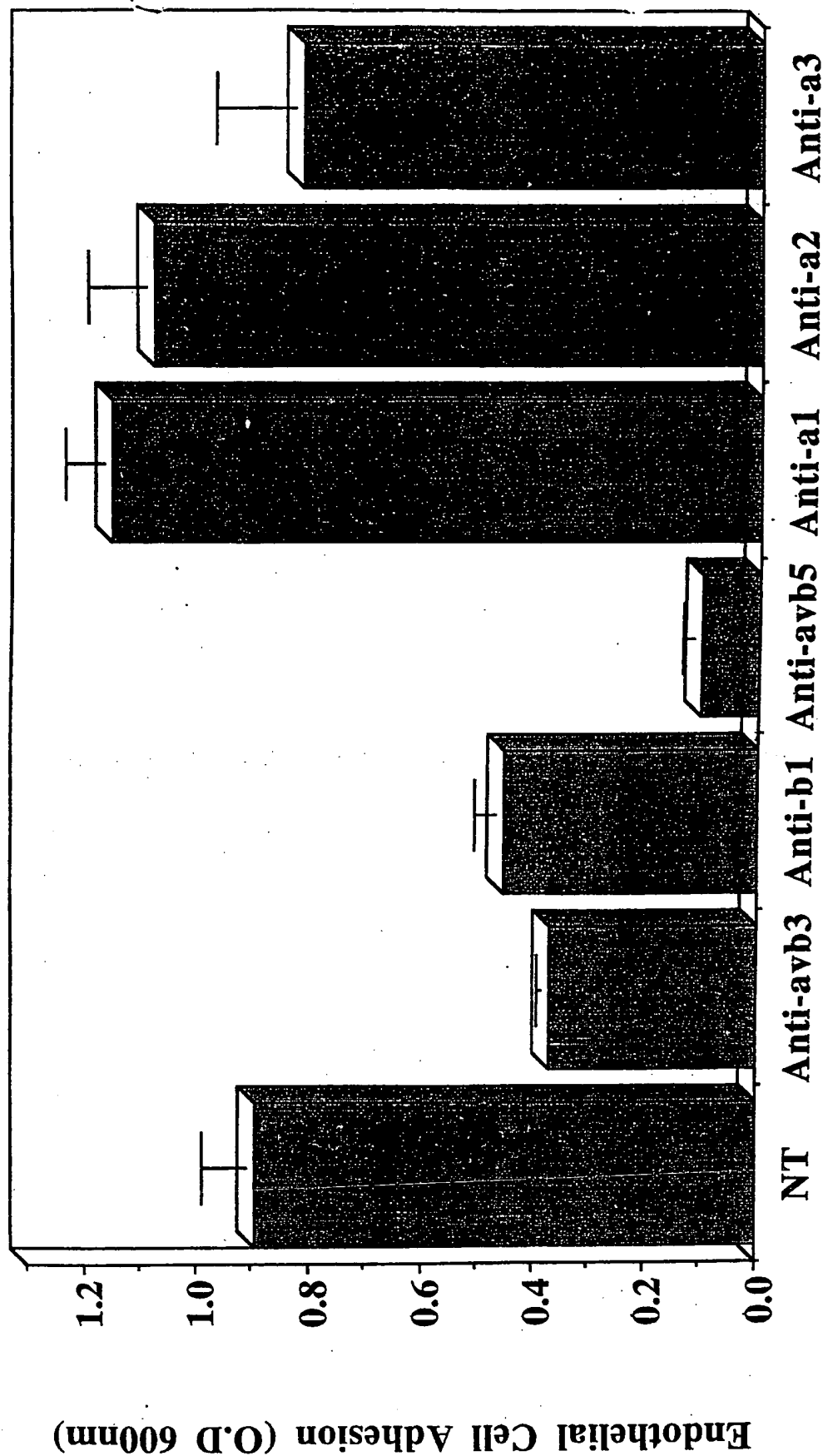
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| | | | | |
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I G Y S F L M H T A A G A E G G G Q S L

1500 1510 1520 1530 1540 1550

FIG. 17f

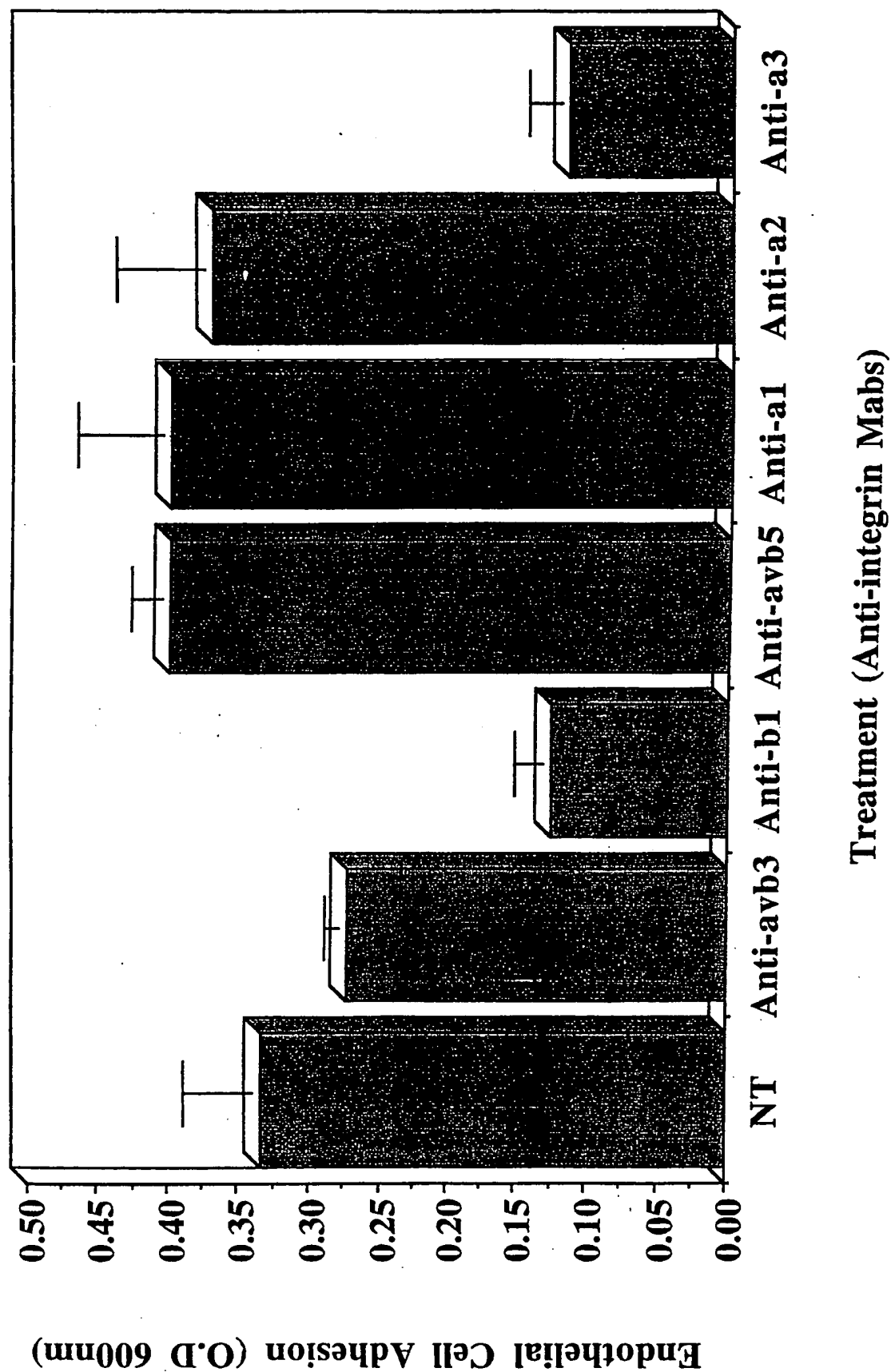
FIG. 17f

FIG. 18



Treatment (Anti-Integrin Mabs)

FIG. 19



SEQUENCE LISTING

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<120> Methods to Inhibit Angiogenesis and Tumor Growth

<130> 94525K2

<140> To be assigned

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Leu Val Thr Arg His Ser Gln Thr Ile Asp Asp Pro Gln Cys Pro Ser
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Gly Thr Lys Ile Leu Tyr His Gly Tyr Ser Leu Leu Tyr Val Gln Gly
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Asn Glu Arg Ala His Gly Gln Asp Leu Gly Thr Ala Gly Ser Cys Leu
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Arg Lys Phe Ser Thr Met Pro Phe Leu Phe Cys Asn Ile Asn Asn Val
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tgc aac ttt gca tca cga aat gac tac tcg tac tgg ctg tcc acc cct      390
Cys Asn Phe Ala Ser Arg Asn Asp Tyr Ser Tyr Trp Leu Ser Thr Pro
              105              110              115

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 Glu Phe Arg Ser Ala Pro Phe Ile Glu Cys His Gly Arg Gly Thr Cys
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Asp	Tyr	Lys	Asp	Asp	Asp	Asp	Lys	Leu	Ala	Val	Ser	Ile	Gly	Tyr	Leu		
			25					30					35				
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Leu	Val	Lys	His	Ser	Gln	Thr	Asp	Gln	Glu	Pro	Met	Cys	Pro	Val	Gly		
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Met	Asn	Lys	Leu	Trp	Ser	Gly	Tyr	Ser	Leu	Leu	Tyr	Phe	Glu	Gly	Gln		
	55					60					65						
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Glu	Lys	Ala	His	Asn	Gln	Asp	Leu	Gly	Leu	Ala	Gly	Ser	Cys	Leu	Ala		
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Arg	Phe	Ser	Thr	Met	Pro	Phe	Leu	Tyr	Cys	Asn	Pro	Gly	Asp	Val	Cys		
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Tyr	Tyr	Ala	Ser	Arg	Asn	Asp	Lys	Ser	Tyr	Trp	Leu	Ser	Thr	Thr	Ala		
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ccg	ctg	ccc	atg	atg	ccc	gtg	gcc	gag	gac	gag	atc	aag	ccc	tac	atc		438
Pro	Leu	Pro	Met	Met	Pro	Val	Ala	Glu	Asp	Glu	Ile	Lys	Pro	Tyr	Ile		
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Ser	Arg	Cys	Ser	Val	Cys	Glu	Ala	Pro	Ala	Ile	Ala	Ile	Ala	Val	His		
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Ser	Gln	Asp	Val	Ser	Ile	Pro	His	Cys	Pro	Ala	Gly	Trp	Arg	Ser	Leu		
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Trp	Ile	Gly	Tyr	Ser	Phe	Leu	Met	His	Thr	Ala	Ala	Gly	Asp	Glu	Gly		
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Gly	Gly	Gln	Ser	Leu	Val	Ser	Pro	Gly	Ser	Cys	Leu	Glu	Asp	Phe	Arg		
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Phe	Gln	Gly	Ser	Pro	Ser	Ala	Asp	Thr	Leu	Lys	Ala	Gly	Leu	Ile	Arg		

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 40 45 50

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 Glu Gly Pro Ala Ile Ala Ile Ala Val His Ser Gln Thr Thr Asp Ile
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Leu	Val	Leu	His	Ser	Gln	Thr	Asp	Gln	Glu	Pro	Thr	Cys	Pro	Leu	Gly														
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/08678

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, PAJ, WPI Data, MEDLINE, CANCERLIT, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 856 184 A (SARRAS JR MICHAEL P ET AL) 5 January 1999 (1999-01-05) column 10, line 39 -column 11, line 12; claims 1-4; figures 7,8,10 column 12, line 14 - line 32	1-4,9-16
X	PRESTAYKO ET AL: "Type IV collagen domains inhibit adhesion and migration of tumor cells and block angiogenesis", PROCEEDINGS OF THE ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH,US,PHILADELPHIA, PA: AACR, VOL. VOL. 39, PAGE(S) 45 XP002118641 abstract	1-4,9-16

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

2 August 2000

Date of mailing of the international search report

22/08/2000

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Noë, V

INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/US 00/08678

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 567 609 A (SARRAS JR MICHAEL P ET AL) 22 October 1996 (1996-10-22) abstract column 1, line 15 - line 24 column 4, line 15 - line 40 column 9, line 8 - line 19	1-12
X	KEFALIDES N A ET AL: "SUPPRESSION OF TUMOR CELL GROWTH BY TYPE IV COLLGEN AND A PEPTIDE FROL THE NC1 DOMAIN OF THE ALPAH3(IV) CHAIN" MEDICINA (BUENOS AIRES), vol. 59, no. 5-2, 1999, pages 553-553, XP002144122 the whole document	1,13
A	HAN JING ET AL: "A cell binding domain from the alpha-3 chain of type IV collagen inhibits proliferation of melanoma cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 33, 1997, pages 20395-20401, XP002144123 ISSN: 0021-9258 page 20400, column 2, paragraph 2; table 2	13-16
A	US 5 766 591 A (BROOKS PETER ET AL) 16 June 1998 (1998-06-16) abstract column 14, line 15 - line 23; examples 7,8,10	1,9,13
A	SETTY SUMAN ET AL: "Interactions of type IV collagen and its domains with human mesangial cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 20, 15 May 1998 (1998-05-15), pages 12244-12249, XP002144124 ISSN: 0021-9258 page 12245, column 1, paragraph 3	3,8,12,16
P,X	WO 99 49885 A (UNIV KANSAS MEDICAL CENTER) 7 October 1999 (1999-10-07) the whole document	1-16
P,X	PETITCLERC E ET AL: "NEW FUNCTION FOR NON- COLLAGENOUS DOMAINS OF HUMAN COLLAGEN" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, 2000, pages 8051-8061, XP002144125 abstract page 8052, column 1, paragraph 2 page 8055 -page 8057; figures 4-6,8	1-4,13-16

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-16 relate to an extremely large number of possible polypeptides. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the polypeptides claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to NC1 monomers of collagen type IV and their fragments.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/08678

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5856184 A	05-01-1999	US 5691182 A US 5567609 A AU 3000895 A EP 0767676 A WO 9600582 A	25-11-1997 22-10-1996 25-01-1996 16-04-1997 11-01-1996
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US 5766591 A	16-06-1998	US 5753230 A AU 709645 B AU 1995295 A CA 2184493 A CN 1151120 A CZ 9602711 A EP 0754059 A FI 963692 A HU 76086 A JP 10500398 T NO 963894 A SK 119096 A WO 9525543 A ZA 9502214 A	19-05-1998 02-09-1999 09-10-1995 28-09-1995 04-06-1997 14-01-1998 22-01-1997 18-09-1996 30-06-1997 13-01-1998 18-11-1996 09-07-1997 28-09-1995 08-02-1996
WO 9949885 A	07-10-1999	AU 3202599 A	18-10-1999

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 October 2000 (12.10.2000)

PCT

(10) International Publication Number
WO 00/59532 A1

(51) International Patent Classification⁷: A61K 38/39

(21) International Application Number: PCT/US00/08678

(22) International Filing Date: 31 March 2000 (31.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/127,391 1 April 1999 (01.04.1999) US

(71) Applicant (for all designated States except US): BIOS-
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(72) Inventors; and

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Drive, Chicago, IL 60606 (US).

(81) Designated States (national): AE, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,
DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,
RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA,
UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent
(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(48) Date of publication of this corrected version:

15 November 2001

(15) Information about Correction:

see PCT Gazette No. 46/2001 of 15 November 2001, Sec-
tion II

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

WO 00/59532 A1

(54) Title: THE USE OF DOMAINS OF TYPE IV COLLAGEN T INHIBIT ANGIOGENESIS AN TUMOUR GROWTH

(57) Abstract: The instant invention provides methods and kits for inhibiting angiogenesis, tumor growth and metastasis, and en-
dothelial cell interactions with the extracellular matrix, involving contacting the tumor, animal tissue, or endothelial cells with an
amount effective to inhibit angiogenesis, tumor growth and metastasis, or endothelial cell interactions with the extracellular matrix
of an antagonist of specific integrin receptors.

THE USE OF DOMAINS OF TYPE IV COLLAGEN TO INHIBIT ANGIOGENESIS AND TUMOUR GROWTH

5

Cross Reference

This application claims priority to U.S. Provisional Application Serial No. 60/127,391 filed April 1, 1999, and is a continuation in part of U.S. Application Serial No. 09/277,665, filed March 26, 1999, which is a continuation in part of U.S. Application Serial No. 09/183,548 filed October 30, 1998, which is a continuation of 10 08/800,965 filed February 18, 1997, now U.S. Patent No. 5,856,184.

Field of the Invention

This invention relates to methods and kits for inhibiting angiogenesis, tumor 15 growth and metastasis, and endothelial cell interactions with the extracellular matrix.

Background of the Invention

Angiogenesis, the process of formation of new blood vessels, plays an important role in physiological processes such as embryonic and postnatal 20 development, as well as in wound repair. Formation of blood vessels can also be induced by pathological processes involving inflammation (e.g., diabetic retinopathy and arthritis) or neoplasia (e.g., cancer) (Folkman, 1985, Perspect, Biol. Med., 29, 10). Neovascularization is regulated by angiogenic growth factors secreted by tumor or normal cells as well as the composition of the extracellular matrix and by the activity of 25 endothelial enzymes (Nicosia and Ottinetti, 1990, Lab. Invest., 63, 115).

During the initial stages of angiogenesis, endothelial cell sprouts appear through gaps in the basement membrane of pre-existing blood vessels (Nicosia and Ottinetti,

1990, *supra*; Schoefl, 1963, Virehous Arch, Pathol. Anat. 337, 97-141; Ausprunk and Folkman, 1977, Microvasc. Res. 14, 53-65; Paku and Paweletz, 1991, Lab. Invest. 63, 334-346). As new vessels form, their basement membrane undergoes complex structural and compositional changes that are believed to affect the angiogenic response (Nicosia, et. al., 1994, Exp. Biology, 164, 197-206). Early planar culture models have shown that basement membrane molecules modulate the attachment, migration, proliferation, and organizational behavior of endothelial cells (Nicosia, et. al., 1994, *supra*). More recent studies with three-dimensional aortic culture models that more closely simulate angiogenic conditions during wound healing in vivo suggest that the basement membrane is a dynamic regulator of angiogenesis, and its function varies according to its molecular components (Nicosia, 1994, *supra*).

A common feature of all solid tumor growth is the requirement for a blood supply. Therefore, numerous laboratories have focused on developing anti-angiogenic compounds based on growth factors and their receptors. While this approach has led to some success, the number of growth factors known to play a role an angiogenesis is large. Therefore, the possibility exists that growth factor antagonists may have only limited use in treating cancer since tumors and associated inflammatory cells likely produce a wide variety of factors that can induce angiogenesis.

In this regard, a strategy that targets a common feature of angiogenesis, such as endothelial cell adhesion to the extracellular matrix (ECM), might be expected to have a profound physiological impact on tumor growth in humans. This notion is supported by the fact that RGD-containing antagonists of the $\alpha v \beta 3$ integrin ECM cell adhesion receptor can block angiogenesis. (U.S. Patent No. 5,766,591) Furthermore, the $\alpha v \beta 3$ integrin is expressed most prominently on cytokine -activated endothelial and smooth

muscle cells and has been shown to be required for angiogenesis. (Varner et al., Cell Adhesion and Communication 3:367-374 (1995); Brooks et al., Science 264:569-571 (1994)). Based on these findings, a potentially powerful new approach to anti-angiogenic therapy might be to specifically target critical regulatory domains within
5 distinct ECM components.

The basement membrane (basal lamina) is a sheet-like extracellular matrix (ECM), which is a basic component of all tissues. The basal lamina provides for the compartmentalization of tissues, and acts as a filter for substances traveling between tissue compartments. Typically the basal lamina is found closely associated with an
10 epithelium or endothelium in all tissues of an animal including blood vessels and capillaries. The basal lamina components are secreted by cells and then self assemble to form an intricate extra-cellular network. The formation of biologically active basal lamina is important to the development and differentiation of the associated cells.

Type IV collagen has been shown to be a major structural component of
15 basement membranes. The protomeric form of type IV collagen is formed as a heterotrimer made up from a number of different subunit chains called $\alpha 1(\text{IV})$ through $\alpha 6(\text{IV})$. Up to now, six genetically distinct α -chains belonging to two classes with extensive homology have been identified, and their relative abundance has been demonstrated to be tissue specific. The type IV collagen heterotrimer is characterized
20 by three distinct structural domains: the non-collagenous (NC1) domain at the carboxyl terminus; the triple helical collagenous domain in the middle region; and the 7S collagenous domain at the amino terminus. (Martin, et. al., 1988, Adv. Protein Chem. 39:1-50; Gunwar, et. al. 1991, J. Biol. Chem. 266:14088-14094).

The ability to express recombinant α (IV) NC1 domains provides the opportunity to study the effect of specific domains on many biological processes, such as angiogenesis, tumor metastasis, cell binding to basement membranes, and assembly of Type IV collagen molecules.

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Summary of the Invention

The instant invention provides methods and kits for inhibiting angiogenesis, tumor growth and metastasis, and endothelial cell interaction with the extracellular matrix, each method comprising contacting the tumor, animal tissue, or endothelial cells with antagonists of specific integrin receptors.

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Brief Description of the Drawings

Figure 1 illustrates the effects of NC1 (Hexamer) and 7S domains of Type IV collagen at a 50 μ g/ml concentration on angiogenesis from mouse thoracic aorta organ cultures.

15 Figure 2 illustrates the effects of 7S domain of Type IV collagen on angiogenesis from mouse thoracic aorta organ cultures. The domain concentrations employed in this experiment were 0 μ g/ml (control); 0.5 μ g/ml; 5 μ g/ml and 50 μ g/ml.

Figure 3 illustrates the effects of NC1 (Hexamer) domain of Type IV collagen on angiogenesis from mouse thoracic aorta organ cultures. The domain concentrations employed in this experiment were 0 μ g/ml (control); 5 μ g/ml and 5 μ g/ml and 50 μ g/ml.

20

Figure 4 are photographs of mouse thoracic aorta segments embedded in Matrigel (EHS basement membrane matrix, Collaborative Biomedical Products, Bedford, MA) at 5 days of culture. Control specimen (0 μ g/ml of NC1 (Hexamer) and 7S domains)

exhibited growth of microvessels from the cultured tissue into the matrix (Figure 4A). In contrast, angiogenesis was inhibited in specimens cultured with 50 $\mu\text{g/ml}$ of 7S domain (Figure 4B) and NC1 (Hexamer) domain (Figure 4C).

Figure 5 is a graphical representation of data demonstrating the in vivo effect of IV
5 injection of recombinant ($\alpha 1$) type IV collagen monomer on angiogenesis using fibrin implants in rats.

Figure 6 is a graphical representation of data demonstrating that the recombinant ($\alpha 1$) and ($\alpha 2$) NC1 monomers inhibit the bFGF-induced increase in angiogenic index in vivo.

10 Figure 7 is a graphical representation of demonstrating the dose response effect of recombinant ($\alpha 2$) NC1 monomer on the bFGF-induced increase in total blood vessel branch points in vivo.

Figure 8 is a graphical representation of data demonstrating the dose response effect of recombinant ($\alpha 2$) NC1 monomer on the bFGF-induced increase in angiogenic index in
15 vivo.

Figure 9 is a graphical representation of data demonstrating the dose response effect of recombinant ($\alpha 2$) NC1 monomer on the bFGF-induced increase in angiogenic index in vivo.

Figure 10 is a graphical representation of data demonstrating the effect of recombinant
20 ($\alpha 1$) and ($\alpha 2$) NC1 monomers on mean CS-1 melanoma tumor weight in vivo.

Figure 11 is a graphical representation of data demonstrating the dose response effect of recombinant ($\alpha 2$) NC1 monomer on mean CS-1 melanoma tumor weight in vivo.

Figure 12 is a graphical representation of data demonstrating the effect of recombinant ($\alpha 1$), ($\alpha 2$), and ($\alpha 4$) NC1 monomers on mean HT1080 tumor weight in vivo.

Figure 13 is a graphical representation of data demonstrating the effect of recombinant ($\alpha 1$), ($\alpha 2$), ($\alpha 3$) and ($\alpha 5$) NC1 monomers on mean HEP-3 tumor weight in vivo.

Figure 14 is a graphical representation of data demonstrating human endothelial cell adhesion to immobilized NC1 α monomers.

- 5 Figure 15 is a graphical representation of data demonstrating the effect of soluble $\alpha 1$ and $\alpha 2$ NC1 monomers on human endothelial cell adhesion to pepsinized collagen type IV.

Figure 16 is a graphical representation of data demonstrating the effect of isolated recombinant NC1 monomers on human endothelial cell migration in vitro.

- 10 Figure 17 A-F provides the sequences of each type IV collagen α chain monomer.

Figure 18 is a graphical representation of data demonstrating the effect of monoclonal antibodies against various integrins on human endothelial cell adhesion to recombinant the ($\alpha 2$) NC1 domain.

- Figure 19 is a graphical representation of data demonstrating human endothelial cell
15 adhesion to the recombinant ($\alpha 1$) NC1 domain.

Description of the Preferred Embodiments

- Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press),
20 *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutscher, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press,

San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique*, 2nd Ed. (R.I. Freshney. 1987. Liss, Inc. New York, NY), and *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

As used herein, the term Type IV collagen domain encompasses the group of
5 molecules including the non-collagenous NC1 domain (Hexamer) and 7S collagenous domains, as well as NC1 α chain monomers.

The invention comprises methods for using Type IV collagen NC1 α -monomers (ie: α 1, α 2, α 3, and α 6), which are defined to include such monomers isolated from any multicellular organism or produced via recombinant protein expression from a gene
10 encoding such a monomer from any multicellular organism, and also to encompass various modifications, additions, and/or deletions to such monomers.

In one aspect, the present invention provides methods and kits for inhibiting angiogenesis in an animal tissue comprising contacting the tumor or animal tissue with an amount effective to inhibit angiogenesis of a polypeptide composition comprising one
15 or more isolated type IV collagen NC1 α chain monomers selected from the group consisting of α 1, α 2, α 3, and α 6 NC1 chain monomers.

In another aspect, the present invention provides methods and kits for inhibiting tumor growth in tissue comprising contacting the tumor or tissue with an amount effective to inhibit tumor growth of a polypeptide composition comprising one or more
20 isolated type IV collagen NC1 α chain monomers selected from the group consisting of α 1, α 2, α 3, and α 6 NC1 chain monomers.

In another aspect, the present invention provides methods and kits for inhibiting tumor metastasis in tissue comprising contacting the tumor or tissue with an amount effective to inhibit metastasis of a polypeptide composition comprising one or more

isolated type IV collagen NC1 α chain monomers selected from the group consisting of $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 6$ NC1 chain monomers.

In a further aspect, the present invention provides methods and kits for inhibiting endothelial cell interactions with the extracellular matrix in tissue comprising
5 contacting the tumor or tissue with an amount effective to inhibit endothelial cell interactions with the extracellular matrix of a polypeptide composition comprising one or more isolated type IV collagen NC1 α chain monomers selected from the group consisting of $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 6$ NC1 chain monomers.

The NC1-encoding domain of each of the six α chain cDNAs has been cloned
10 into a vector for recombinant protein expression as previously described (Sado et al., Kidney Intl. 53:664-671 (1998), incorporated by reference herein in its entirety). The vectors are used to stably transfect human kidney 293 cells, which produce the recombinant protein. The DNA and deduced amino acid sequences of the recombinant type IV collagen alpha chain monomers produced as described are shown in Figure
15 17A-F. The first 17 amino acids correspond to a BM40 signal sequence (which is cleaved from the mature protein), to facilitate protein secretion. All the secreted proteins (ie: mature proteins) start with the sequence APLA followed by the affinity tag, DYKDDDDK at the amino terminus. This tag facilitates purification and identification of the material, and does not interfere with biological activity of the
20 recombinant NC1 α chain monomers.

The type IV collagen NC1 α chain monomers can be produced by any method known in the art, including using recombinant DNA technology or biochemical peptide synthesis technology, or by isolating the NC1 domains from animal sources, such as from basement membrane sources such as bovine lens capsule and bovine kidney glomeruli.

(Peczon et al., Exp. Eye Res. 30:155-165 (1980); Langeveld et al., J. Biol. Chem. 263:10481-10488 (1988); Gunwar et al., J. Biol. Chem. 266:14088-14094 (1991))

In practicing the invention, the amount or dosage range of type IV collagen NC1 α chain monomers employed is one that effectively inhibits angiogenesis, tumor
5 growth, tumor metastasis, and/or endothelial cell-extracellular matrix interactions. An inhibiting amount of NC1 α chain monomers that can be employed ranges generally between about 0.01 $\mu\text{g/kg}$ body weight and about 10 mg/kg body weight, preferably ranging between about 0.05 $\mu\text{g/kg}$ and about 5 mg/kg body weight.

The NC1 α chain monomers may be administered by any suitable route,
10 including orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intraarterial, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally. In preferred embodiments, the
15 NC1 α chain monomers are administered intravenously or subcutaneously.

The NC1 α chain monomers may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (e.g., solutions, suspensions, or emulsions). The NC1 α chain monomers of the invention may be applied in a variety of solutions. Suitable solutions for use in accordance with the invention are sterile,
20 dissolve sufficient amounts of the NC1 α chain monomers, and are not harmful for the proposed application.

The NC1 α chain monomers may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

For administration, the NC1 α chain monomers are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanolic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Example 1 – In Vitro Effect on Angiogenesis

With modifications, the procedures of Nicosia and Ottinetti (1990), *supra*, and Nicosia, et. al. (1994), *supra*, were utilized for experiments designed to test the effect of Type IV collagen on angiogenesis under *in vitro* conditions. The model has been used to study the effects of growth factors and extracellular matrix molecules on the

angiogenic response and employs aortic rings cultures in three-dimensional collagen gels under serum-free conditions. These experiments are outlined below.

A. Methods

Experiments were performed with 1-3 month old Swiss Webster male mice.

- 5 Following anesthesia, the thoracic aorta was excised under aseptic conditions and transferred to sterile MCDB 131 sterile growth medium (Clonetics, San Diego, CA) containing antibiotics. Fat was dissected away from the aorta and approximately six to eight 1 mm thoracic segments were obtained from each specimen. Segments were transferred to 48 well tissue culture plates. The wells of these plates were layered with
- 10 100 microliters of Matrigel (EHS basement membrane, Collaborative Biomedical Products, Bedford, MA) prior to transfer of the aortic segments. The Matrigel was diluted 1:1 with MCDB 131 growth medium prior to use. The segments were centered in the wells and an additional 100 microliters of Matrigel was then placed over the specimens. The aortic segments were therefore embedded in the basement membrane
- 15 matrix. Each well then received 300 microliters of MCDB 131 growth medium. The plates were placed in an incubator maintained at 37° C with 5% CO₂. Specimens were observed daily over a 7 day period. Newly growing microvessels were counted using an inverted phase microscope at various times during the culture period, but data is expressed at 3 and 5 days of culture. To test for the effect of Type IV collagen on
- 20 angiogenesis, domains at known concentrations were mixed with the Matrigel and with the MCDB 131 growth medium. Fresh MCDB 131 growth medium (plus and minus collagen domains) was changed every 3 days.

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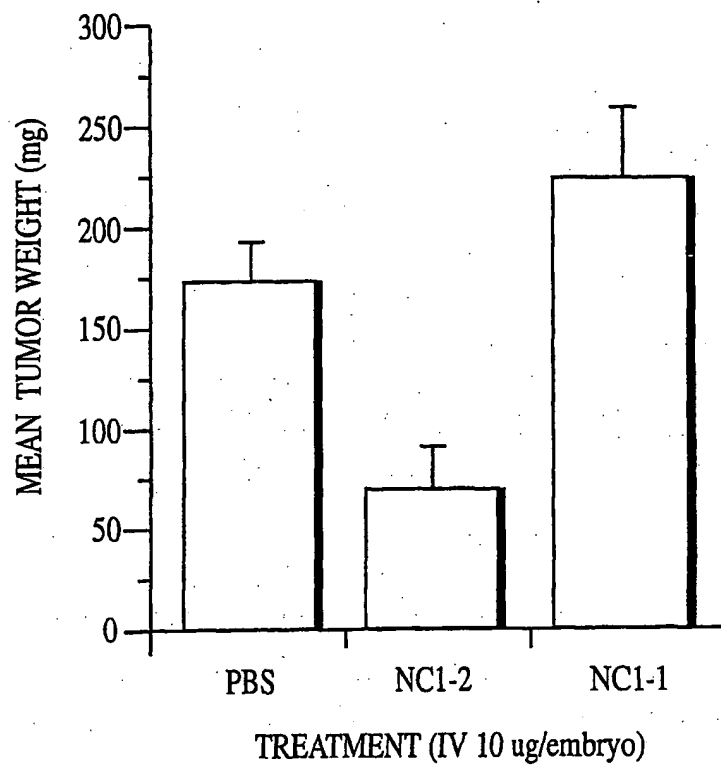


FIG. 10

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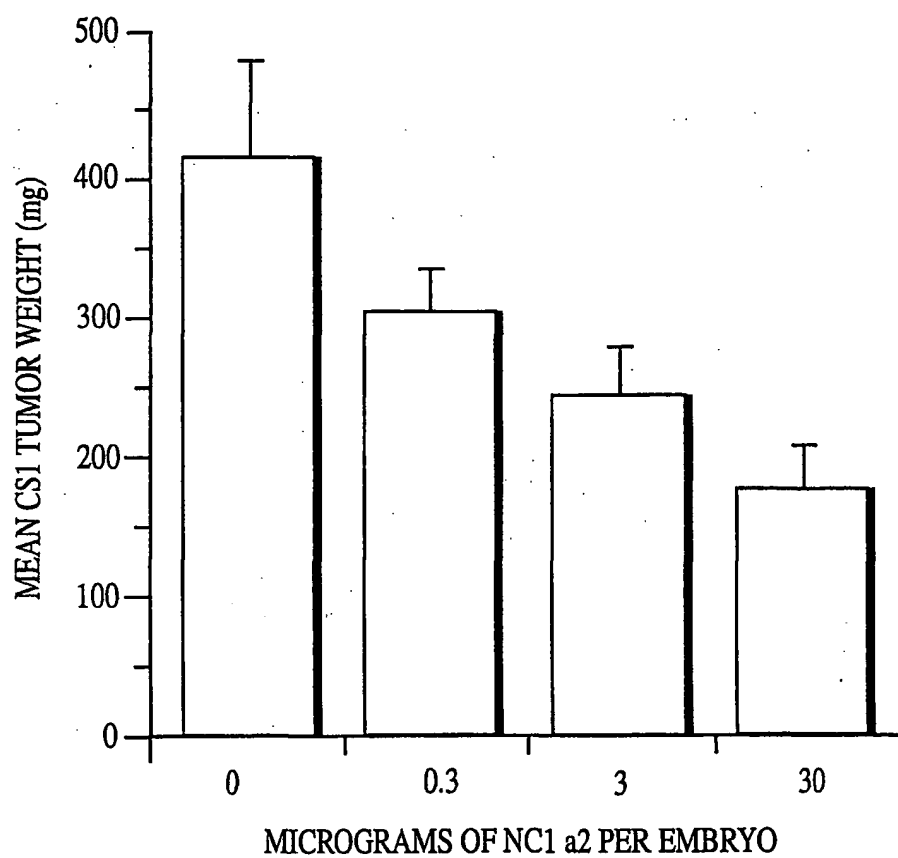


FIG. 11

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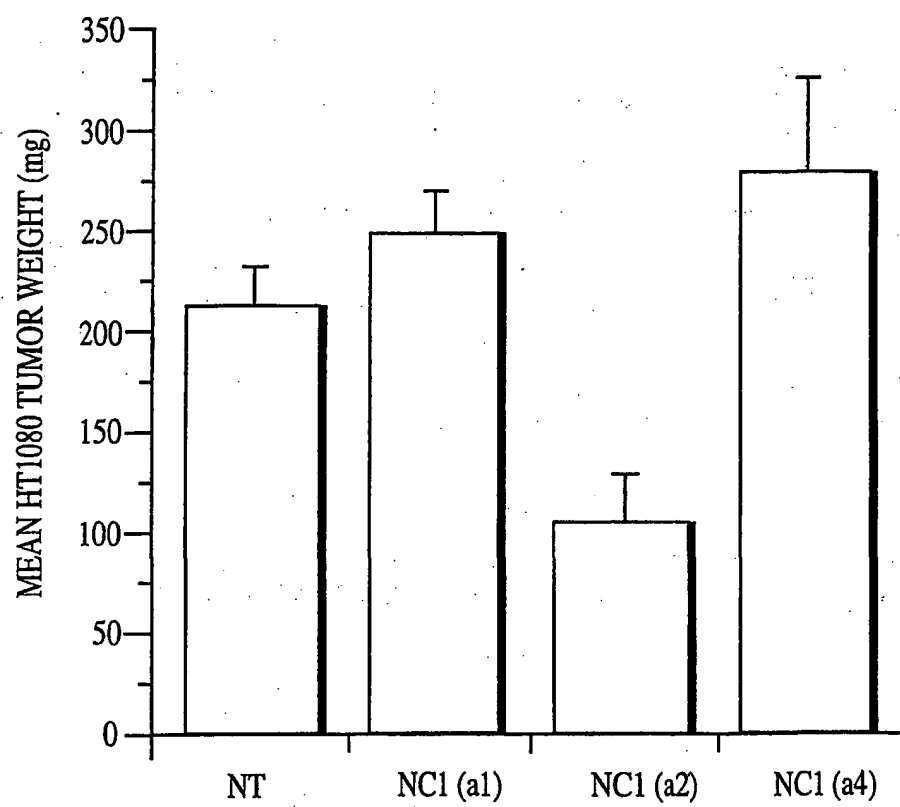


FIG. 12

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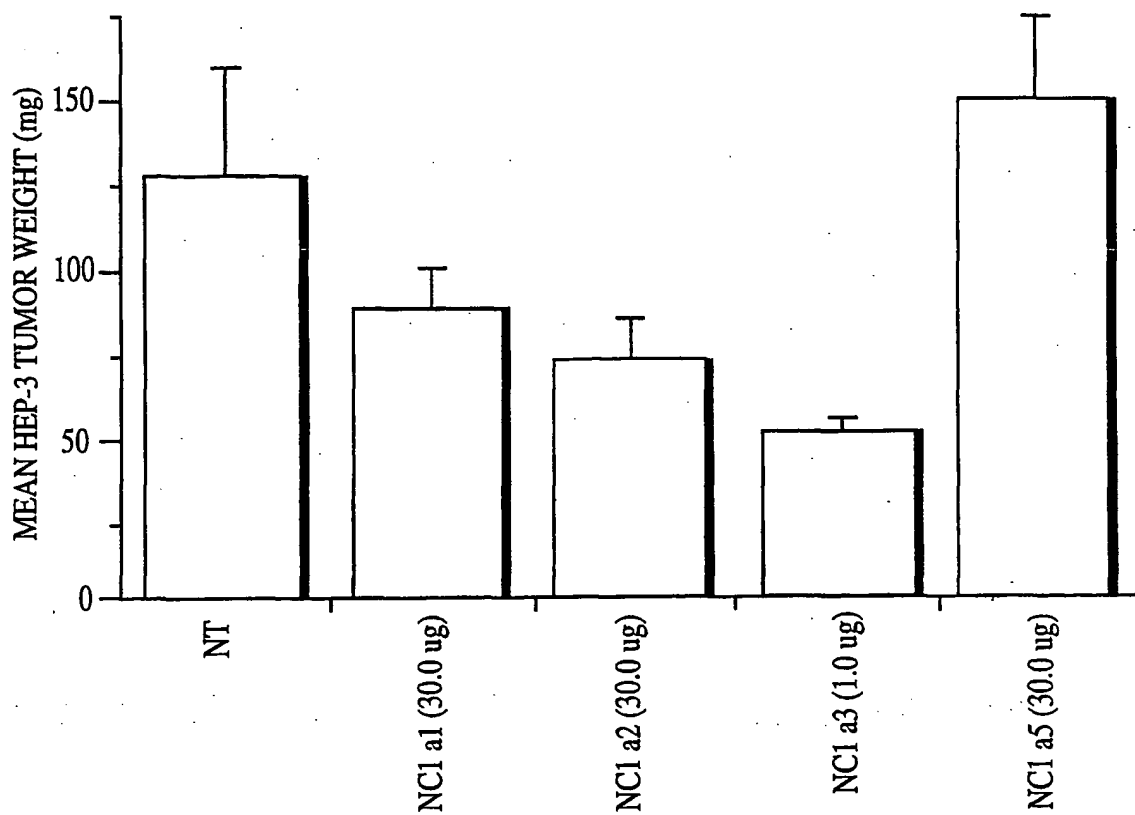


FIG. 13

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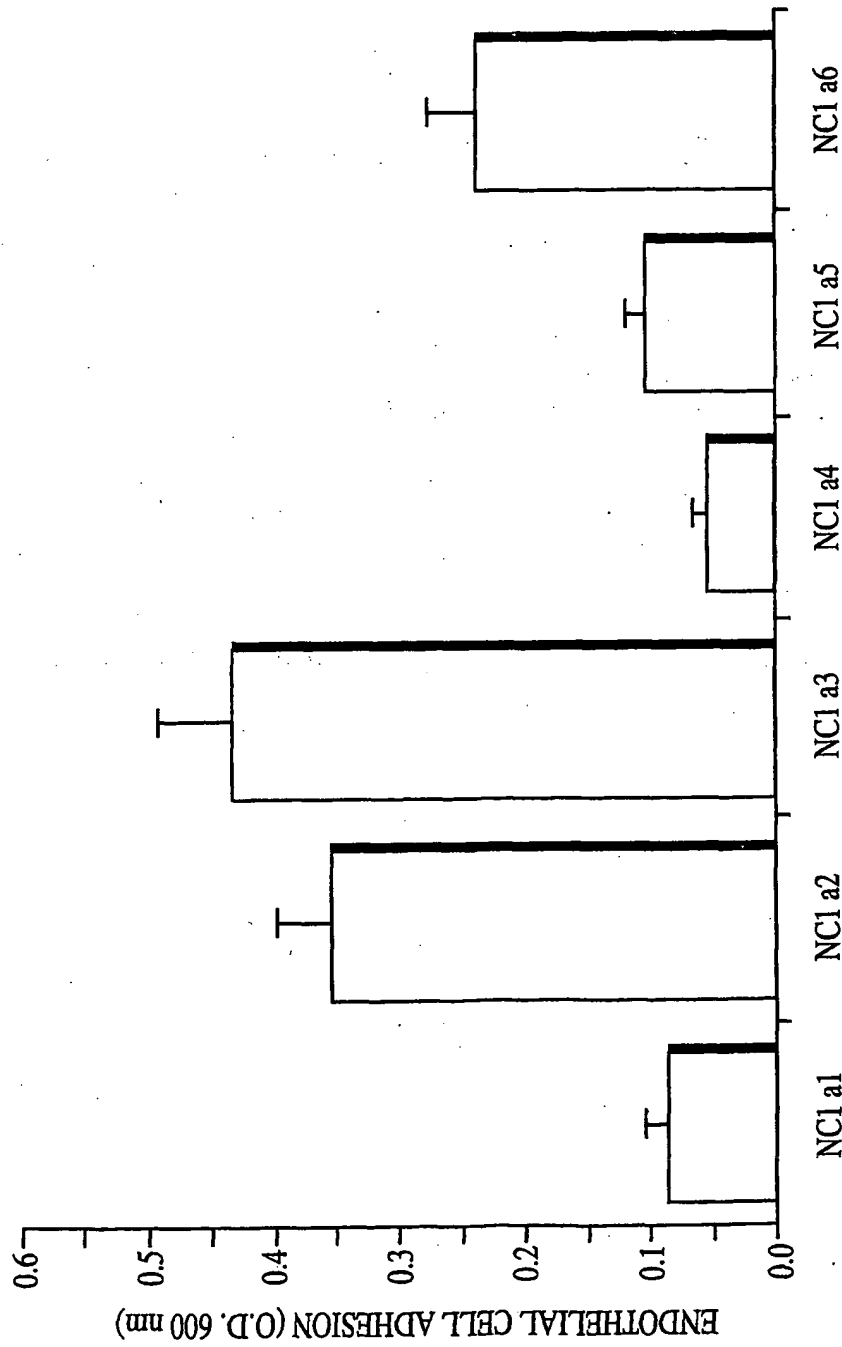


FIG. 14

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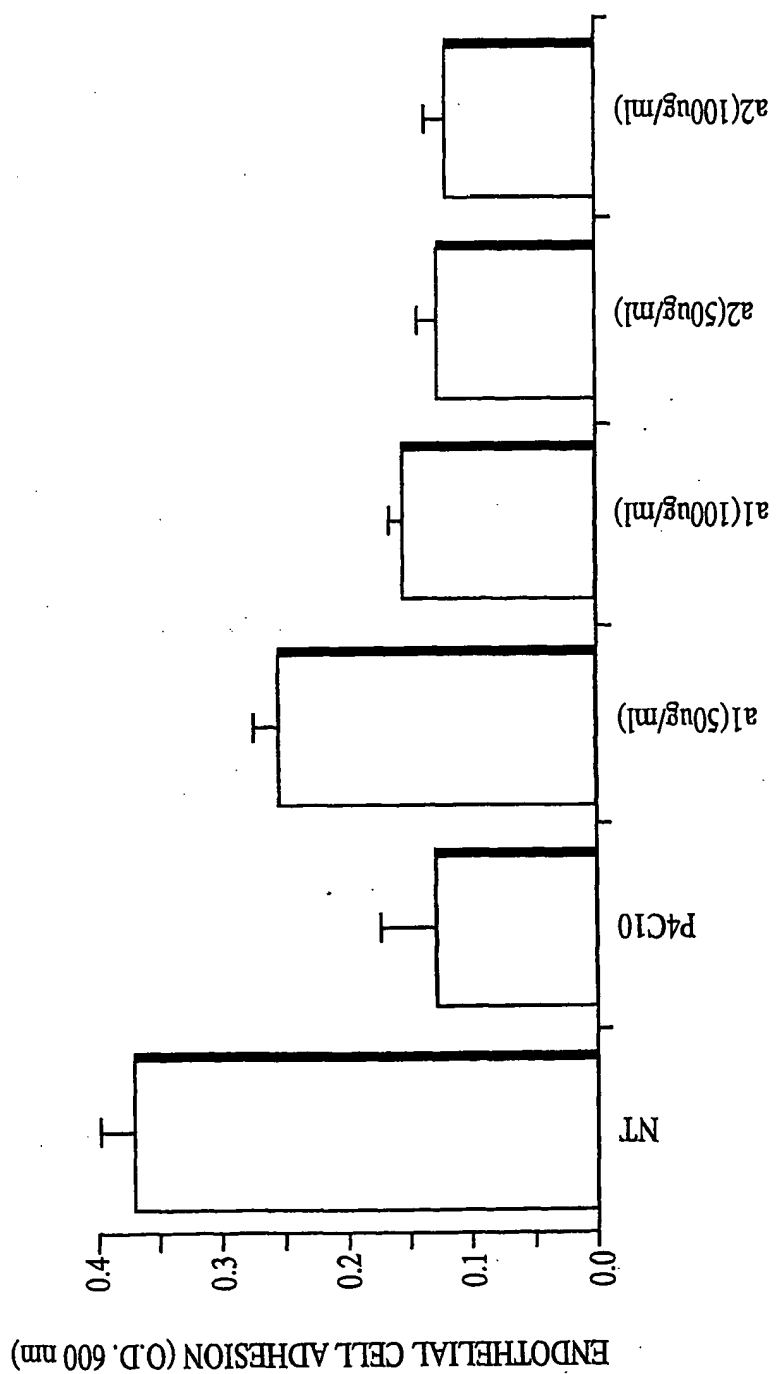


FIG. 15

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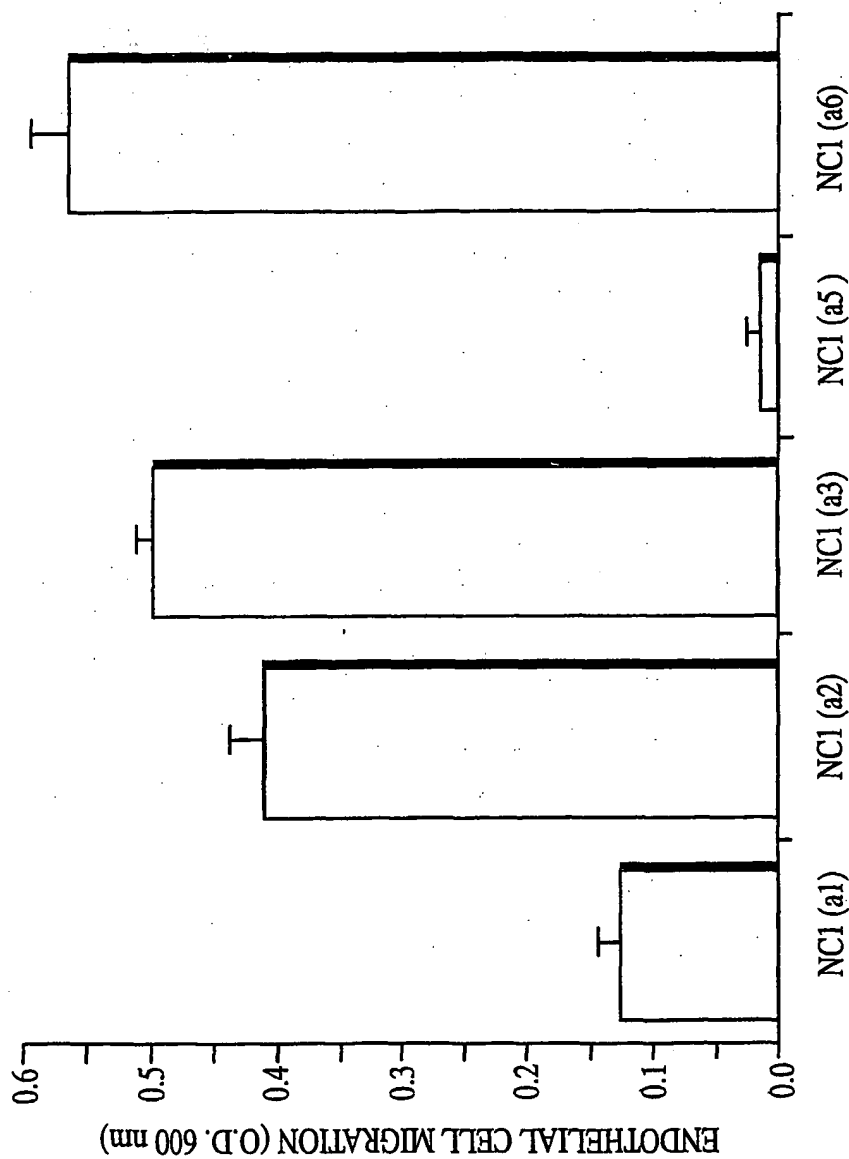


FIG. 16

A. $\alpha 1$ (IV) NC1

17/30

900 910 920 930 940 950
| | | | |
CTGCCGCCTGCCTGCCTGCCACTGAGGGTTCCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCACTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | |
GACAAGCTAGCATCTGTGATCACGGCTTCCTTGTGACCAGGCATAGTCAAACAATAGAT
D K L A S V D H G F L V T R H S Q T I D

1080 1090 1100 1110 1120 1130
| | | | |
GACCCACAGTGTCTCTTCTGGGACCAAAATTCTTTACCACGGGTACTCTTTGCTCTACGTG
D P Q C P S G T K I L Y H G Y S L L Y V

1140 1150 1160 1170 1180 1190
| | | | |
CAAGGCAATGAACGGGCCCATGGCCAGGACTTGGGCACGGCCGGCAGCTGCCTGCGCAAG
Q G N E R A H G Q D L G T A G S C L R K

1200 1210 1220 1230 1240 1250
| | | | |
TTCAGCACAAATGCCCTTCTCTGTTCTGCAATATTAACAACGTGTGCAACTTTGCATCACGA
F S T M P F L F C N I N N V C N F A S R

1260 1270 1280 1290 1300 1310
| | | | |
AATGACTACTCGTACTGGCTGTCCACCCCTGAGCCCATGCCCATGTCAATGGCACCCATC
N D Y S Y W L S T P E P M P M S M A P I

1320 1330 1340 1350 1360 1370
| | | | |
ACGGGGGAAAACATAAGACCATTTATTAGTAGGTGTGCTGTGTGTGAGGCGCCTGCCATG
T G E N I R P F I S R C A V C E A P A M

1380 1390 1400 1410 1420 1430
| | | | |
GTGATGGCCGTGCACAGCCAGACCATTCAGATCCCACCGTGCCCCAGCGGGTGGTCCTCG
V M A V H S Q T I Q I P P C P S G W S S

1440 1450 1460 1470 1480 1490
| | | | |
CTGTGGATCGGCTACTCTTTTGTGATGCACACCAGCGCTGGTGCAGAAGGCTCTGGCCAA
L W I G Y S F V M H T S A G A E G S G Q

1500 1510 1520 1530 1540 1550
| | | | |
GCCCTGGCGTCCCCCGGCTCCTGCCTGGAGGAGTTTAGAAGTGCGCCATTCATCGAGTGT

FIG. 17a

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A L A S P G S C L E E F R S A P F I E C

1560 1570 1580 1590 1600 1610
| | | | | |
CACGGCCGTGGGACCTGCAATTACTACGCAAACGCTTACAGCTTTTGGCTCGCCACCATA
H G R G T C N Y Y A N A Y S F W L A T I

1620 1630 1640 1650 1660 1670
| | | | | |
GAGAGGAGCGAGATGTTCAAGAAGCCTACGCCGTCCACCTTGAAGGCAGGGGAGCTGCGC
E R S E M F K K P T P S T L K A G E L R

1680 1690 1700 1710 1720 1730
| | | | | |
ACGCACGTCAGCCGCTGCCAAGTCTGTATGAGAAGAACATAATGAAGCCTGACTCAGCTA
T H V S R C Q V C M R R T - -

1740 1750 1760 1770 1780 1790
| | | | | |
CCGCGGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTG

FIG. 17a

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B. $\alpha 2$ (IV) NC1

900 910 920 930 940 950
CTGCCGCCTGCCTGCCACTGAGGGTTCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCACTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
GACAAGCTAGCCGTCAGCATCGGCTACCTCCTGGTGAAGCACAGCCAGACGGACCAGGAG
D K L A V S I G Y L L V K H S Q T D Q E

1080 1090 1100 1110 1120 1130
CCCATGTGCCCCGGTGGGCATGAACAACTCTGGAGTGGATACAGCCTGCTGTACTTCGAG
P M C P V G M N K L W S G Y S L L Y F E

1140 1150 1160 1170 1180 1190
GGCCAGGAGAAGGCGCACAAACCAGGACCTGGGGCTGGCGGGCTCCTGCCTGGCGCGGTTC
G Q E K A H N Q D L G L A G S C L A R F

1200 1210 1220 1230 1240 1250
AGCACCATGCCCCTTCCTGTACTGCAACCCTGGTGTGTCTGCTACTATGCCAGCCGGAAC
S T M P F L Y C N P G D V C Y Y A S R N

1260 1270 1280 1290 1300 1310
GACAAGTCCTACTGGCTCTCTACCACTGCGCCGCTGCCCATGATGCCCGTGGCCGAGGAC
D K S Y W L S T T A P L P M M P V A E D

1320 1330 1340 1350 1360 1370
GAGATCAAGCCCTACATCAGCCGCTGTTCTGTGTGTGAGGCCCCGGCCATCGCCATCGCG
E I K P Y I S R C S V C E A P A I A I A

1380 1390 1400 1410 1420 1430
GTCCACAGTCAGGATGTCTCCATCCCACACTGCCAGCTGGGTGGCGGAGTTTGTGGATC
V H S Q D V S I P H C P A G W R S L W I

1440 1450 1460 1470 1480 1490
GGATATTCTTCCTCATGCACACGGCGGGAGACGAAGCGGTGGCCAATCACTGGTG
G Y S F L M H T A A G D E G G G Q S L V

1500 1510 1520 1530 1540 1550

FIG. 17b

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C. $\alpha 3$ (IV) NC1

900 910 920 930 940 950
| | | | | |
CTGCCGCCTGCCTGCCACTGAGGGTCCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCGCTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | | |
GACAAACGTGGAGACAGTGGATCACCTGCAACCTGGACAACGAGAGGCTTTGTCTTCACC
D K R G D S G S P A T W T T R G F V F T

1080 1090 1100 1110 1120 1130
| | | | | |
CGACACAGTCAAACCACAGCAATTCCTTCATGTCCAGAGGGGACAGTGCCACTCTACAGT
R H S Q T T A I P S C P E G T V P L Y S

1140 1150 1160 1170 1180 1190
| | | | | |
GGGTTTTCTTTCTTTTGTACAAGGAAATCAACGAGCCACGGACAAGACCTTGGAAC
G F S F L F V Q G N Q R A H G Q D L G T

1200 1210 1220 1230 1240 1250
| | | | | |
CTTGGCAGCTGCCTGCAGCGATTTACCACAATGCCATTCTTATTCTGCAATGTCAATGAT
L G S C L Q R F T T M P F L F C N V N D

1260 1270 1280 1290 1300 1310
| | | | | |
GTATGTAATTTTGCATCTCGAAATGATTATTTCATACTGGCTGTCAACACCAGCTCTGATG
V C N F A S R N D Y S Y W L S T P A L M

1320 1330 1340 1350 1360 1370
| | | | | |
CCAATGAACATGGCTCCCATTACTGGCAGAGCCCTTGAGCCTTATATAAGCAGATGCACT
P M N M A P I T G R A L E P Y I S R C T

1380 1390 1400 1410 1420 1430
| | | | | |
GTTTGTGAAGGTCCTGCGATCGCCATAGCCGTTTCACAGCCAAACCACTGACATTCTCTCA
V C E G P A I A I A V H S Q T T D I P P

1440 1450 1460 1470 1480 1490
| | | | | |
TGTCCTCACGGCTGGATTTCTCTCTGGAAGGATTTTCATTTCATCATGTTTACAAGTGCA
C P H G W I S L W K G F S F I M F T S A

1500 1510 1520 1530 1540 1550

FIG. 17c

SUBSTITUTE SHEET (RULE 26)

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GGTTCTGAGGGCGCCGGGCAAGCACTGGCCTCCCCCGGCTCCTGCCTGGAAGAATCCGA
G S E G A G Q A L A S P G S C L E E F R

1560 1570 1580 1590 1600 1610
GCCAGCCCATTCTAGAAATGTCATGGAAGAGGAACGTGCAACTACTATTCAAATTCCTAC
A S P F L E C H G R G T C N Y Y S N S Y

1620 1630 1640 1650 1660 1670
AGTTTCTGGCTGGCTTCATTAAACCCAGAAAGAATGTTTCAGAAAGCCTATTCCATCAACT
S F W L A S L N P E R M F R K P I P S T

1680 1690 1700 1710 1720 1730
GTGAAAGCTGGGGAATTAGAAAAATAATAAGTCGCTGTCAGGTGTGCATGAAGAAAAGA
V K A G E L E K I I S R C Q V C M K K R

1740 1750 1760 1770 1780 1790
CACTGAGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGCTGATCAGCCTCGAC
H -

FIG. 17c

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D. $\alpha 4$ (IV)NC1

900 910 920 930 940 950
| | | | | |
CTGCCGCTGCCTGCCTGCCACTGAGGGTTCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCGCTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | | |
GACAAGCCTGGATACCTCGGTGGCTTCTCTGGTTCTCCACAGTCAGACGGACCAGGAG
D K P G Y L G G F L L V L H S Q T D Q E

1080 1090 1100 1110 1120 1130
| | | | | |
CCCACCTGCCCCCTGGGCATGCCAGGCTCTGGACTGGGTATAGTCTGTTATACCTGGAA
P T C P L G M P R L W T G Y S L L Y L E

1140 1150 1160 1170 1180 1190
| | | | | |
GGGCAAGAGAAAGCTCACAATCAAGACCTTGGTCTGGCAGGGTCTTGCCTTCCCGTATTT
G Q E K A H N Q D L G L A G S C L P V F

1200 1210 1220 1230 1240 1250
| | | | | |
AGCACGCTGCCCTTTGCCTACTGCAACATCCACCAGGTGTGCCACTATGCCAGAGAAAC
S T L P F A Y C N I H Q V C H Y A Q R N

1260 1270 1280 1290 1300 1310
| | | | | |
GACAGATCCTACTGGCTGGCCAGCGCTGCGCCCCCTCCCATGATGCCACTCTCTGAAGAG
D R S Y W L A S A A P L P M M P L S E E

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A I R P Y V S R C A V C E A P A Q A V A

1380 1390 1400 1410 1420 1430
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V H S Q D Q S I P P C P Q T W R S L W I

1440 1450 1460 1470 1480 1490
| | | | | |
GGGTATTCATTCTGATGCACACAGGAGCTGGGGACCAAGGAGGAGGGCAGGCCCTTATG
G Y S F L M H T G A G D Q G G G Q A L M

1500 1510 1520 1530 1540 1550

FIG. 17d

[illegible]

SUBSTITUTE SHEET (RULE 26)

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E. $\alpha 5$ (IV) NC1

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| | | | |
CTGCCGCCTGCCTGCCACTGAGGGTCCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCGCTAGCTGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | |
GACAAAGGTCCCCCTGGAACCTCCTCTGTTCACATGGATTTCTTATTACACGCCACAGC
D K G P P G T S S V A H G F L I T R H S

1080 1090 1100 1110 1120 1130
| | | | |
CAGACAACGGATGCACCACAATGCCCACAGGGAACACTTCAGGTCTATGAAGGCTTTTCT
Q T T D A P Q C P Q G T L Q V Y E G F S

1140 1150 1160 1170 1180 1190
| | | | |
CTCCTGTATGTACAAGGAAATAAAAGAGCCCACGGTCAAGACTTGGGGACGGCTGGCAGC
L L Y V Q G N K R A H G Q D L G T A G S

1200 1210 1220 1230 1240 1250
| | | | |
TGCCTTCGTCGCTTTAGTACCATGCCTTTCATGTTCTGCAACATCAATAATGTTTGCAAC
C L R R F S T M P F M F C N I N N V C N

1260 1270 1280 1290 1300 1310
| | | | |
TTTGCTTCAAGAAATGACTATTCTTACTGGCTCTCTACCCAGAGCCCATGCCAATGAGC
F A S R N D Y S Y W L S T P E P M P M S

1320 1330 1340 1350 1360 1370
| | | | |
ATGCAACCCCTAAAGGGCCAGAGCATCCAGCCATTCAATTAGTCGATGTGCAGTATGTGAA
M Q P L K G Q S I Q P F I S R C A V C E

1380 1390 1400 1410 1420 1430
| | | | |
GCTCCAGCTGTGGTGATCGCAGTTCACAGTCAGACGATCCAGATTCCTCCATTGCTCCTCAG
A P A V V I A V H S Q T I Q I P H C P Q

1440 1450 1460 1470 1480 1490
| | | | |
GGATGGGATTCTCTGTGGATTGGTTATTCCTTCATGATGCATACAAGTGCAGGGGCAGAA
G W D S L W I G Y S F M M H T S A G A E

1500 1510 1520 1530 1540 1550

FIG. 17e

[illegible]

SUBSTITUTE SHEET (RULE 26)

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F. $\alpha 6$ (IV) NC1

900 910 920 930 940 950
| | | | | |
CTGCCGCTGCCTGCCACTGAGGGTTCCCAGCACCATGAGGGCCTGGATCTTCTTT
M R A W I F F

960 970 980 990 1000 1010
| | | | | |
CTCCTTTGCCTGGCCGGGAGGGCTCTGGCAGCCCCACTAGCCGACTACAAGGACGACGAT
L L C L A G R A L A A P L A D Y K D D D

1020 1030 1040 1050 1060 1070
| | | | | |
GACAAGCTAGCGAGCATGAGAGTGGGCTACACGTTGGTAAAGCACAGCCAGTCGGAACAG
D K L A S M R V G Y T L V K H S Q S E Q

1080 1090 1100 1110 1120 1130
| | | | | |
GTGCCCCCGTGTCCCATCGGGATGAGCCAGCTGTGGGTGGGGTACAGCTTACTGTTTGTG
V P P C P I G M S Q L W V G Y S L L F V

1140 1150 1160 1170 1180 1190
| | | | | |
GAGGGGCAAGAGAAAGCCCACAACCAGGACCTGGGCTTTGCTGGCTCCTGTCTGCCCCGC
E G Q E K A H N Q D L G F A G S C L P R

1200 1210 1220 1230 1240 1250
| | | | | |
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F S T M P F I Y C N I N E V C H Y A R R

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AATGATAAATCTTACTGGCTCTCCACTACCGCCCCCTATCCCCATGATGCCCGTCAGCCAG
N D K S Y W L S T T A P I P M M P V S Q

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A V H S Q D I T I P Q C P L G W R S L W

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| | | | | |
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I G Y S F L M H T A A G A E G G G Q S L

1500 1510 1520 1530 1540 1550

FIG. 17f

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V S P G S C L E D F R A T P F I E C S G

1560 1570 1580 1590 1600 1610
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A R G T C H Y F A N K Y S F W L T T V E

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E R Q Q F G E L P V S E T L K A G Q L H

1680 1690 1700 1710 1720 1730
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T R V S R C Q V C M K S L -

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FIG. 17f

SUBSTITUTE SHEET (RULE 26)

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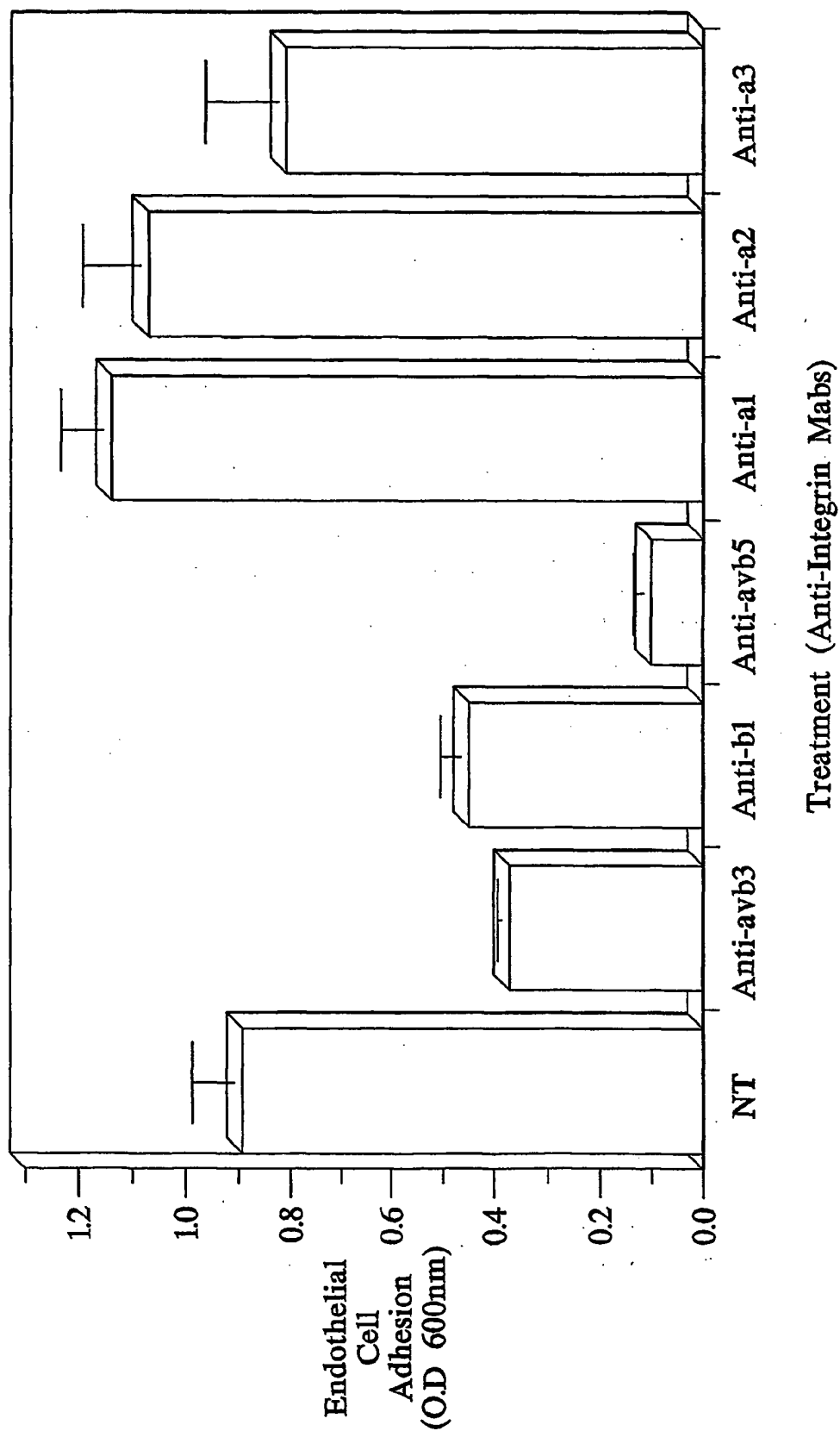
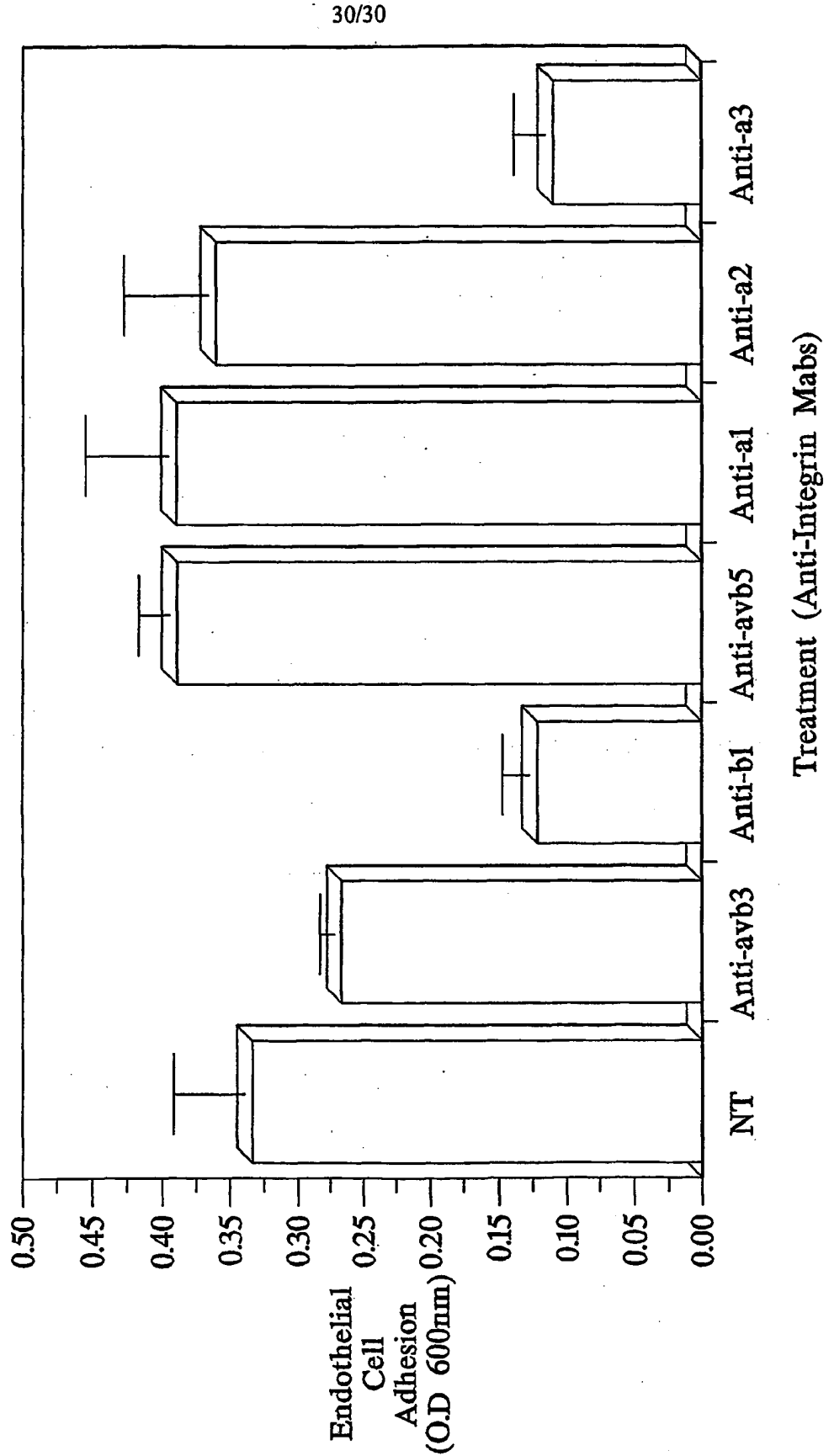
FIG. 18

FIG. 19



SEQUENCE LISTING

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 185 190 195

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INTERNATIONAL SEARCH REPORT

Internal Application No

PCT/US 00/08678

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, PAJ, WPI Data, MEDLINE, CANCERLIT, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 856 184 A (SARRAS JR MICHAEL P ET AL) 5 January 1999 (1999-01-05) column 10, line 39 -column 11, line 12; claims 1-4; figures 7,8,10 column 12, line 14 - line 32	1-4,9-16
X	PRESTAYKO ET AL: "Type IV collagen domains inhibit adhesion and migration of tumor cells and block angiogenesis", PROCEEDINGS OF THE ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH,US,PHILADELPHIA, PA: AACR, VOL. VOL. 39, PAGE(S) 45 XP002118641 abstract	1-4,9-16
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

2 August 2000

Date of mailing of the international search report

22/08/2000

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Noë, V

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/08678

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 567 609 A (SARRAS JR MICHAEL P ET AL) 22 October 1996 (1996-10-22) abstract column 1, line 15 - line 24 column 4, line 15 - line 40 column 9, line 8 - line 19	1-12
X	KEFALIDES N A ET AL: "SUPPRESSION OF TUMOR CELL GROWTH BY TYPE IV COLLAGEN AND A PEPTIDE FROM THE NC1 DOMAIN OF THE ALPHA3(IV) CHAIN" MEDICINA (BUENOS AIRES), vol. 59, no. 5-2, 1999, pages 553-553, XP002144122 the whole document	1,13
A	HAN JING ET AL: "A cell binding domain from the alpha-3 chain of type IV collagen inhibits proliferation of melanoma cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 33, 1997, pages 20395-20401, XP002144123 ISSN: 0021-9258 page 20400, column 2, paragraph 2; table 2	13-16
A	US 5 766 591 A (BROOKS PETER ET AL) 16 June 1998 (1998-06-16) abstract column 14, line 15 - line 23; examples 7,8,10	1,9,13
A	SETTY SUMAN ET AL: "Interactions of type IV collagen and its domains with human mesangial cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 20, 15 May 1998 (1998-05-15), pages 12244-12249, XP002144124 ISSN: 0021-9258 page 12245, column 1, paragraph 3	3,8,12, 16
P,X	WO 99 49885 A (UNIV KANSAS MEDICAL CENTER) 7 October 1999 (1999-10-07) the whole document	1-16
P,X	PETITCLERC E ET AL: "NEW FUNCTION FOR NON-COLLAGENOUS DOMAINS OF HUMAN COLLAGEN" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, 2000, pages 8051-8061, XP002144125 abstract page 8052, column 1, paragraph 2 page 8055 -page 8057; figures 4-6,8	1-4, 13-16

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-16 relate to an extremely large number of possible polypeptides. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the polypeptides claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to NC1 monomers of collagen type IV and their fragments.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/08678

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5856184 A	05-01-1999	US 5691182 A US 5567609 A AU 3000895 A EP 0767676 A WO 9600582 A	25-11-1997 22-10-1996 25-01-1996 16-04-1997 11-01-1996
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WO 9949885 A	07-10-1999	AU 3202599 A	18-10-1999

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